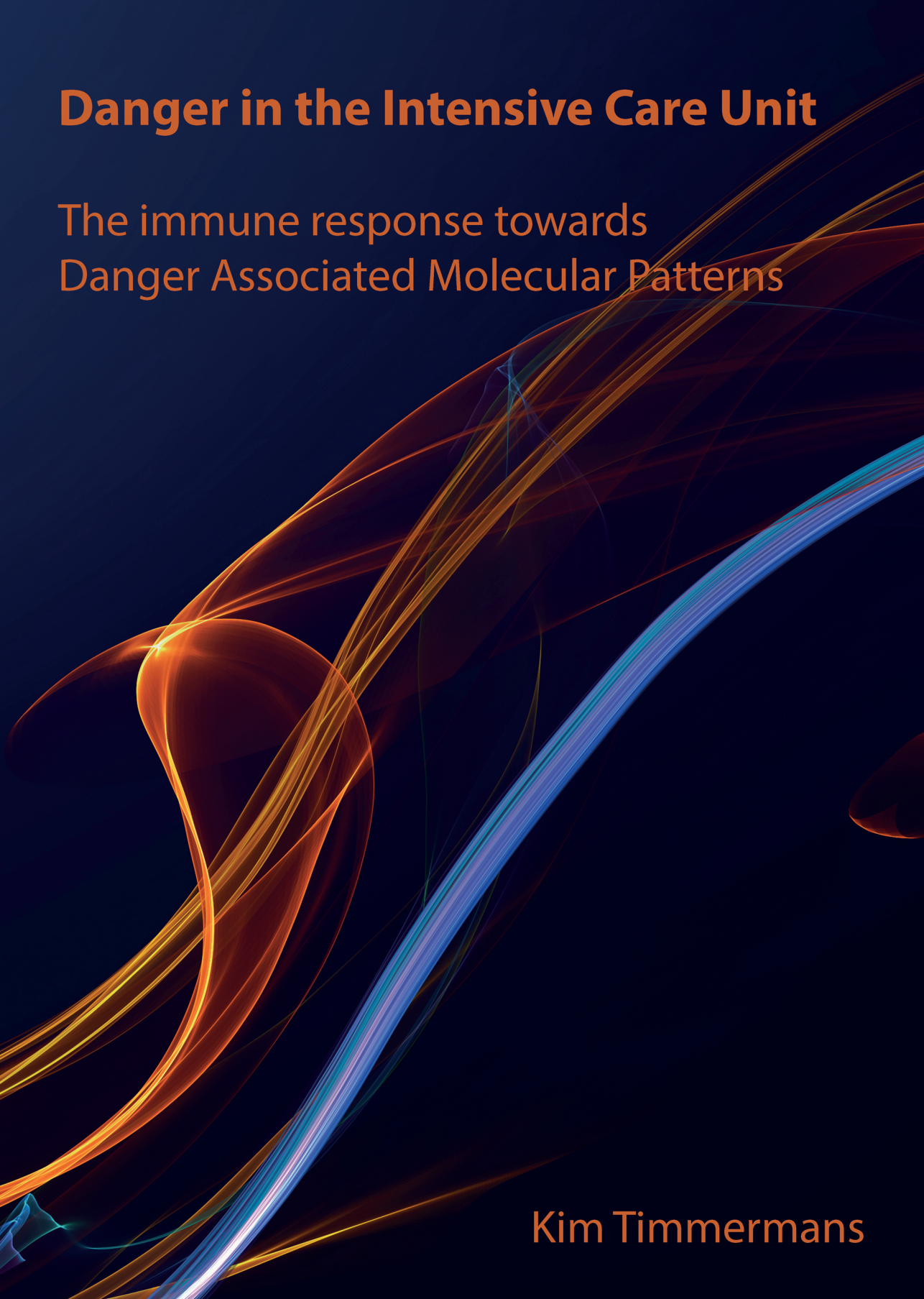
# Danger in the Intensive Care Unit

## The immune response towards Danger Associated Molecular Patterns



Kim Timmermans

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The immune response towards Danger Associated Molecular Patterns

**Kim Timmermans**

## Colofon

The research presented in this thesis was performed at the Departments of Anesthesiology and Intensive Care Medicine, Radboud University Medical Center, Nijmegen, the Netherlands.

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# Danger in the Intensive Care Unit

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*To those who made me who I am*

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# Chapter 1

## Introduction and outline of this thesis

Adapted from: "Danger in the Intensive Care Unit: DAMPs in critically ill patients"

Kim Timmermans, Matthijs Kox, Gert Jan Scheffer, Peter Pickkers

*Shock*; Epub 2015 Oct 17

The immune system is a complex network consisting of many elements primarily aimed to protect the human body from invading pathogens. An important feature of the immune system is the ability to distinguish between entities it should react to, such as bacteria and viruses, and those it should ignore, such as the host's healthy tissue [1]. Cells of the innate immune system, such as macrophages and dendritic cells, discriminate between these and mount an initial non-specific immunologic response [2]. Furthermore, these antigen presenting cells (APCs) subsequently instruct lymphocytes that orchestrate a specific adaptive immune response [2]. If these systems function as intended, an effective and appropriate immune response is mounted that eliminates the threat, but does not cause collateral damage to organs. The immune system detects invading pathogens through recognition of so-called "Pathogen Associated Molecular Patterns" (PAMPs) by Pattern Recognition Receptors (PRRs). PAMPs are parts of microbes, such as lipopolysaccharide (LPS), a major component of the outer membrane of gram-negative bacteria, or e.g. β-glucan, a cell-wall component of fungi. Examples of PRRs include Toll-Like receptors (TLRs), NOD-like receptors (NLRs), and C-type lectin Receptors (CLRs), to which specific PAMPs bind. However, it has become clear that PRRs can also be activated by so-called so-called "Danger-Associated Molecular Patterns" (DAMPs). In the studies described in this thesis, we focused on immunologic roles and mechanisms of action of DAMPs in critically ill patients. Next to a pro-inflammatory response, triggering of the immune system could also lead to an anti-inflammatory response, called immunoparalysis, that leaves patients susceptible for secondary infections. We have studied those aspects in several groups of critically ill patients, namely those suffering from trauma, cardiac arrest, leukemia, and sepsis. Furthermore, we performed pre-clinical studies in mice on the role of DAMPs and underlying mechanisms of ventilator-induced inflammation, and assessed interactions between ligation of different PRRs in vitro. Finally, immunologically inactive biomarkers were studied in trauma patients.

## The Danger Model and DAMPs

Clearly, the immune system can discriminate between self and non-self to a great extent [1], however, as presented by Polly Matzinger in their 'Danger Model', it is assumed that the immune system does not primarily discriminate between self and non-self, but rather between safe and dangerous [3, 4]. This model describes how APCs are activated by danger signals, or DAMPs, that are released by injured, threatened, or dead cells, or originate from the extracellular matrix. Of interest,

for example apoptosis does not result in the release of DAMPs as leakage of cell components, intracellular substances, or other molecules is prevented in this controlled manner of cell death [5]. However, if cells die in an uncontrolled fashion, such as necrosis or rupture by physical force, cellular contents and debris, some of which are DAMPs, are released into the surrounding tissue and/or circulation [5]. In a similar fashion, the extracellular matrix can be disrupted, resulting in the release of components which act as DAMPs. Cells can also actively release DAMPs when under threat [6]. DAMPs can be constitutively expressed or inducible as well as intracellular or secreted [5]. Taken together, it is clear that DAMPs that activate or influence immune responses can be released in a multitude of situations and in many different ways, thereby forming a large group of biologically active molecules, that as such could also serve as biomarkers or therapeutic targets.

Several criteria, some of which overlap, have been put forward that molecules should fulfil to qualify as DAMP [6, 7]. These can be summarized into: 1. The molecule is released rapidly following a triggering event. 2. The purified molecule induces an inflammatory response at physiological concentrations, and is particularly potent *in vivo*. 3. Selective elimination or neutralization of the molecule inhibits the biological activity of dead cells in in vitro and in vivo assays. 4. The molecule has both chemotactic as well as APC-activating effects. However, not all molecules generally accepted as DAMPs fulfil all these criteria.

A broad range of DAMPs meeting these criteria have been described in the literature, indicating that the Danger Model, originally described as a theoretical model [3, 4], plays an important role in regulation of immune responses. As a result, in recent years, the involvement of DAMPs in immune responses has gained increasing attention. This is of great relevance in critically ill patients, in whom trauma- or surgery-related cell damage, hypoxia, ischemia, and infections may result in extensive release of DAMPs. As many patients in the intensive care unit suffer from immune system-related complications [8], DAMPs could play an important role in the prognosis of these patients and represent possible therapeutic targets. As such, further understanding of the mechanisms behind DAMP release and DAMP-induced immune responses is warranted.

Hereby, we provide an overview of several well-described DAMPs (High Mobility Group Box 1, heat shock proteins, s100 proteins, and nucleic acids) and their effects on the immune system. Furthermore, we discuss the role of DAMPs as markers and therapeutic targets in several conditions frequently encountered in the intensive care unit, such as sepsis, trauma, ventilator-induced lung injury (VILI), and cardiac arrest. An overview of observational human studies and animal intervention studies relevant to intensive care-related conditions is provided in

Tables 1 and 2, respectively. Moreover, the mechanisms of release and receptor binding capacity of DAMPs are summarized in Figure 1.

## Well-described DAMPs

### High Mobility Group Box 1

High Mobility Group Box 1 (HMGB1) is an archetypal DAMP, previously referred to as HMG-1 or amphoterin [9]. The "High Mobility Group" of nuclear proteins was discovered more than 40 years ago and owes its name to rapid electrophoresis mobility properties in polyacrylamide gels [10]. HMGB1 is an ubiquitous nuclear protein, functioning as a DNA chaperone under physiological conditions [11]. Moreover, it reduces protein aggregation induced by heat or chemical stress in the cytoplasm [12]. However, HMGB1 also exhibits cytokine-like properties when secreted, as it triggers innate immune responses, mediates inflammatory response in later stages [13], and influences immunological processes like autophagy and apoptosis [14].

HMGB1 can be secreted in two ways: actively by inflammatory cells such as macrophages [13], and passively by necrotic and apoptotic cells [15]. In the latter case, HMGB1 diffuses out of the nucleus when membrane integrity is lost [15, 16]. HMGB1 consists of two homologous DNA-binding domains, the A-box and B-box, of which the B-box is related to the cytokine-like properties of HMGB1 [17]. Extracellular HMGB1 acts via a multitude of pathways, among others the binding of several pattern recognition receptors and subsequent NF-κB activation. HMGB1 acts as a ligand for various pattern recognition receptors, including RAGE, TLR2, TRL4 and TLR9 [18-21]. Moreover, it interacts with other pro-inflammatory mediators, such as LPS, LTA and CpG, to enhance TLR-4-, TLR2- and TLR9-mediated immune responses, respectively [22-24].

### S100 proteins

The family of S100 proteins, or calgranulins, consists of 24 members [25] and their name is derived from their solubility in 100% ammonium sulfate solution [26]. Intracellularly, S100 proteins play an important role in the regulation of various processes, such as cell proliferation and differentiation, apoptosis, Ca2+ homeostasis and energy metabolism [25]. However, several S100 family members, e.g. S100A8 (also known as calgranulin A or MRP-8), S100A9 (Calgranulin B or MRP-14, which can form a dimer with S100A8 extracellularly) [27], S100A12 (calgranulin C or EN-RAGE) [28], and S100B [29] can be released extracellularly and subsequently exert pro-inflammatory effects [30, 31]. S100A8 and A100A9 are secreted by

granulocytes, monocytes, keratinocytes, and epithelial cells in inflammatory settings [30, 32-34]. S100A12 is exclusively expressed in granulocytes [35]. Passive release of S100 proteins as a result of necrosis occurs in conjunction with release of other DAMPs in this setting [36, 37]. Interestingly, S100 proteins cannot be secreted via the classical Golgi-route due to their structure that lacks the corresponding signal sequence [38]. Their active release, for example from activated monocytes, occurs via an alternative tubulin-dependent pathway [38].
After release, S100A8/9 interacts both with TLRs and RAGE to exhibit its effects, while S100A12 and S100B interact primarily with RAGE [39-42]. Both will result in NFκB-mediated production of pro-inflammatory cytokines [39, 41]. It appears likely that other receptors can be activated as well [43]. Both proteins also upregulate ICAM-1 and VCAM-1 expression on endothelial cells [39, 44]. Moreover, both S100A8/A9 and S100A12 exert antibacterial/antiparasitic activity [45, 46]. Although S100A8/A9 and S100A12 are released under similar conditions, no interactions between both have been demonstrated [35]. S100B is a brain-specific S100 protein, that has both neurotrophic and proapoptotic effects [47].

**Heat-shock proteins**

Heat-shock proteins (HSPs) are molecular chaperones present in the cytosol that are induced by both hyper- and hypothermia, but also by a variety of other stress factors (e.g. UV radiation, heavy metals, pathogens, and physiological stresses) [48]. They can prevent cell death through a mechanism known as the heat shock response [49]. This response mainly comprises binding of unfolded, misfolded, or mutated proteins for transport to and refolding in the endoplasmic reticulum [50, 51]. Dendritic cells can distinguish stressed apoptotic cells from non-stressed apoptotic cells by detecting the presence of HSPs on the plasma membrane [52]. HSP family members are named after their molecular weight (in kDa), of which the HSP70 family is the most extensively studied [53].
The stress-induced HSP70, both known as "HSP70" and "HSP72" in the literature [53], can be released into the circulation within minutes following an insult [54]. HSPs can be released both passively and actively. Passively, HSPs originate from necrotic or damaged cells. However, HSPs can also be released actively in the absence of necrotic or damaged cells, for example by immune cells [55-57], or in psychological stress situations [58]. Exosomes [55, 59] and surface membrane lipid rafts [60, 61] play an important role in the active release of HSPs.
After release, HSP70 is able to bind to both TLR2 and TLR-4 [62] in a CD14-dependent manner [63, 64]. Moreover, HSP70 binds to CD36 [65], CD40 [66], CD91 [67], Lox-1 [68] and SR-A [69], on a range of cells, mainly those of the innate immune system [65, 69, 70]. On the

other hand, HSP70 does not bind to T-lymphocytes [71]. Interestingly, HSP70 also inhibits HMGB1 release, thereby attenuating the HMGB1-induced inflammatory response and tissue damage [72].

**Nucleic acids**

Naturally, nucleic acids are present in all cells. The nucleus contains DNA coding for all proteins through transcription of mRNA and subsequent translation. Moreover, the endosymbiotic theory suggests that mitochondria originate from free-living bacteria, and as such, mitochondria have their own DNA (mtDNA) [73]. Nuclear DNA (nDNA), RNA, and mtDNA can bind to TLRs and as such stimulate the production of pro-inflammatory cytokines [74-76].

Nucleic acids are released into the circulation after rupture or necrosis of cells [77]. However, active release, both spontaneous and during cell death, has also been described [78]. The exact mechanisms behind active release of nucleic acids remain to be further elucidated.

Double stranded DNA is able to bind to RIG-I or DAI (DNA-dependent activator of IFN-regulatory factors), mRNA to TLR3 [76, 79], and TLR-9 specifically recognizes unmethylated CpG sequences present in high amounts in mtDNA [80, 81]. As such, the release of nucleic acids exert inflammatory effects. However, as the receptors involved are mainly present intracellularly, internalization by cells such as APCs is often necessary for nucleic acids to function as a DAMP [82]. As such, it has been suggested that for nucleic acids to function as a DAMP, release of other DAMPs, such as HMGB1, is required. Furthermore, nucleic acids also affect the activity of other DAMPs, thereby increasing their inflammatory potential [82].

**Other DAMPs**

Hyaluronan or Hyaluronic Acid is an important extracellular matrix component, existing in both a low molecular weight (LMW) and high molecular weight (HMW) form [83]. The latter is an important molecule in cell adhesion and tissue architecture [84]. Under inflammatory conditions, HMW hyaluronan is broken down into LMW hyaluronan and/or LMW hyaluronan de novo synthesis occurs through hyaluronan sytnhase-3 (HAS-3) [83]. LMW hyaluronan mainly exerts pro-inflammatory effects through binding of TLR2 and TLR-4 [85], whereas HMW hyaluronan exerts anti-inflammatory properties [85, 86].

Micro- and nanoparticles are produced by many different cell types in response to cell activation or cell death and are also suggested to function as DAMPs [87]. These particles are formed if components, e.g. single-stranded RNA and protamine, are mixed or if monocytes are stimulated with unesterified cholesterol [88, 89]. The

immune system can distinguish the size of the particles in order to initiate an antiviral (nano) or antibacterial/antifungal (micro) directed immune response [88].

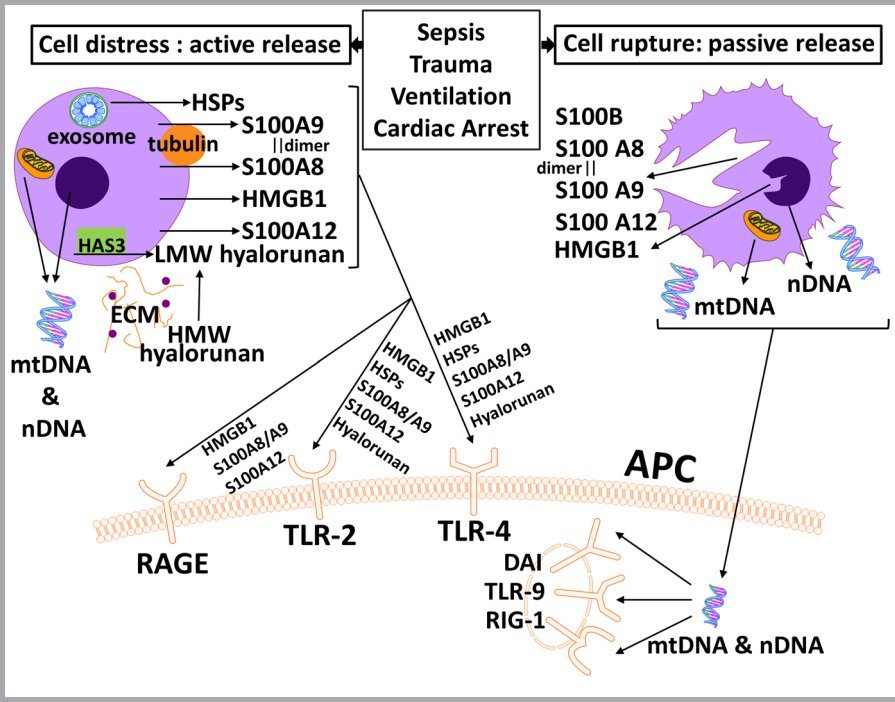


***Figure 1 – Mechanisms of DAMP release and receptor ligation in intensive-care related conditions*** *RAGE = receptor for advanced glycation endproducts; TLR = Toll-like receptor; DAI = DNA-dependent activator of IFN-regulatory factors; RIG-1 = retinoic acid-inducible gene 1*

## DAMPs in intensive care-related conditions

### Sepsis

Sepsis is the leading cause of death in the intensive care unit [90], generally characterized by an early hyperinflammatory phase and a subsequent immunosuppressive phase, known as 'immunoparalysis', that can last for days or even weeks [8]. More recent evidence suggests that the hyperinflammatory and immunoparalytic phases develop simultaneously [91, 92]. Especially in the hyperinflammatory reaction in sepsis, PAMPs play an important role in the inflammatory response. However, DAMPS are released as well, contributing to initiation and/or propagation of the immune response in sepsis [93]. Previous studies in primates suggest that ongoing tissue damage and DAMP release occurs

during lethal bacterial sepsis [94], making it difficult to distinguish between PAMP- and DAMP-related responses in those patients.

HMGB1 is one of the most well-studied DAMPs in sepsis. Plasma HMGB1 level correlates with the degree of organ dysfunction that occurs in late-phase sepsis and may discriminate between survivors and non-survivors in both murine endotoxemia and human sepsis [13, 95]. Furthore, in mice, neutralization of HMGB1 dose-dependently reverses endotoxic shock and established sepsis, prevents organ injury, and increases survival [13, 96]. However, in a prospective study assessing HMGB1 release over time in patients with sepsis, severe sepsis, or sepsis shock, the HMGB1 plasma level (measured up to day 6) was markedly lower in septic patients who died compared with survivors [97]. As these data are contradictory, one might speculate that HMGB-1 shows dual release characteristics: higher in dying subjects (vs. survivors) in acute sepsis and lower in a more persistent ("chronic") sepsis scenario. HSPs, of which HSP70 is the most well-studied in this context, appear to impact the development of sepsis and septic shock, and genetic variations in the HSP genes have been demonstrated to influence outcome of sepsis [98-100]. However, animal studies that have investigated HSP70 neutralization in sepsis have yielded contradictory results, therefore no definite conclusions on the role of HSP70 in sepsis can be drawn [101, 102]. S100 proteins S100A8/A9 also appear to be involved in the pathophysiology of sepsis. Their plasma level is increased in patients with severe sepsis as well as in healthy volunteers after administration of endotoxin, an experimental model mimicking many hallmarks of the systemic inflammatory response observed in septic patients [103]. Furthermore, S100A8/A9 deficient mice exhibit a reduced systemic inflammatory response after endotoxin administration [41], and inhibition of S100A8/A9 prevents lethality in murine sepsis [41]. Plasma DNA levels (both nuclear and mitochondrial) are elevated in septic patients as well, and correlate with mortality [104]. Likewise, plasma mtDNA level increases during the course of lethal sepsis in primates [94], although an observational study in sepsis patients did not confirm these results [105]. In a recent murine study, administration of DNAses (to remove free nucleic acids from the circulation) resulted in decreased coagulation and inflammation, suppression of organ damage, and improved outcome in a cecal ligation and puncture sepsis model [161]. These findings suggest a detrimental role of nucleic acids in sepsis. Furthermore, plasma hyalorunan level is increased in septic shock patients, are higher in non-survivors compared with survivors, and correlate with inflammatory cytokine levels in those patients [106]. Interestingly, the administration of HMW hyaluronan appears to reduce sepsis-induced lung injury in rats [107].

**Trauma**

Multiple trauma often elicits a systemic inflammatory response syndrome and can also result in the development of immunoparalysis [108, 109]. Apart from the direct effects of the initial injury itself, the subsequent immunological complications are partly responsible for trauma-induced morbidity and mortality. Due to the trauma-induced cell damage, large amounts of DAMPs are released into the circulation, which, in the absence of invading pathogens are believed to be responsible for the observed immunological responses [110].

HMGB1 appears to be an important DAMP in the inflammatory response observed following trauma and/or severe bleeding. HMGB1 level is increased early after trauma, and non-survivors demonstrate a higher plasma level compared with survivors [111]. Furthermore, animal studies have revealed that HMGB1 elicits an immune response after trauma in a TLR-4-dependent manner [112]. Neutralization of HMGB1 improves outcome in various animal models of trauma and hemorrhagic shock [113-116]. Heat shock proteins are also implicated in trauma, and are suggested to exert beneficial effects, as a higher HSP70 plasma level correlates with increased survival in these patients [54]. Furthermore and possibly related to this, they appear to play a neuroprotective role in spinal cord injury [117]. Furthermore, several S100 proteins are released following trauma. For example, S100A8 and S100A9 induce an inflammatory response following damage of peripheral nerves [118]. Interestingly, blunt trauma survivors demonstrate higher plasma S100A8/A9 level compared with non-survivors, suggesting a protective role of S100 proteins following trauma [119]. S100B is released after traumatic brain injury and appears a promising marker for the diagnosis of brain damage or spinal cord injury [36, 120]. Release of mtDNA is also suggested to play a role in trauma. The plasma level of mtDNA is increased following trauma [121, 122]. Furthermore, in rats, intravenous administration of mitochondrial DAMPS, consisting of mtDNA and formyl peptides, resulted in a systemic inflammatory reaction and lung injury in a TLR9-dependent manner [122]. Finally, plasma hyalorunan concentration was higher in trauma patients compared with healthy controls and correlated with markers for impaired coagulation [123]. To date, no studies have been undertaken to investigate the effects of neutralization of S100 proteins, mtDNA, or hyaluronan in (animal models of) trauma.

**Ventilator-induced Lung Injury**

Mechanical ventilation is an essential part of intensive care medicine, although it may also cause ventilator-induced lung injury (VILI) [124]. VILI is characterized by a sterile inflammatory response in the lungs resulting in tissue damage that may

| | HMGB1 | HSP |
|---|---|---|
| **Sepsis** | Sepsis: non-survivors plasma HMGB1↑ vs. survivors [13]<br><br>Sepsis: plasma HMGB1 over time: survivors↓, non-survivors↑ [95]<br><br>(Severe) sepsis+septic shock: plasma HMGB1↑ during 1 week after admittance, plasma HMGB1↓ non-survivors [97] | Severe sepsis: plasma HSPA12B↑ vs. sepsis/SIRS<br>Non survivors plasma HSPA12B↑ vs. survivors [99]<br><br>HSP70 gene polymorphisms influence outcome [100] |
| **Trauma** | Trauma patients with hemorrhagic shock: Plasma HMGB1↑[113]<br><br>Severe trauma patients: Plasma HMGB1↑, correlated with injury severity, SIRS, and complement activation. Non-survivors plasma HMGB1↑ vs survivors [111] | Trauma patients, ventilated 2+ days, Injury Severity Score ≥16: Plasma HSP72↑: low plasma HSP72: survival↓ [54] |
| **VILI** | Short-term MV (5h): BALF HMGB1 not altered, Long-term MV (days): BALF HMGB1↑, no difference between infected and non-infected lung in unilateral ventilator-associated pneumonia [132] | Acute lung injury/ARDS: HSP72 plasma and pulmonary edema fluid↑.<br>Preserved alveolar epithelial fluid clearance: HSP72↑ [135] |
| **Cardiac arrest** | Cardiac arrest patients: HMGB1↑ in cerebrospinal fluid of patients with worse neurological outcome. No differences in serum [150] | Cardiac arrest: Plasma HSP70↑, correlated with immunoparalysis [154]<br><br>Acute myocardial infarction: serum HSP70↑, correlated with IL-6 and IL-8 [157] |

***Table 1 – Observational human studies on DAMPs in intensive care-related conditions***

*ARDS=acute respiratory distress syndrome; BALF=bronchoalveolar lavage fluid*

| S100 proteins | Nucleic acids |
|---|---|
| Severe sepsis or LPS injection in healthy volunteers: Plasma S100A8/A9↑ Peritonitis: Abdominal fluid S100A8/A9↑ vs. plasma [103] | Sepsis and septic shock mtDNA = healthy controls [105] |
| Chronic spinal cord injury (SCI): S100A12, S100A8, S100A9 in wound fluid↓ vs non-SCI patients [120] | Trauma: Plasma mtDNA↑ [121, 122] |
| ARDS: S100A12 expression in lung↑, in BALF↑ [137, 138]<br><br>LPS inhalation in healthy volunteers: S100A12 in BALF↑ [137]<br><br>S100A8/A9 to S100A12 ratio in BALF: different between chronic and acute lung disease [138] | Not investigated. |
| Cardiac arrest: plasma S100A12↑, correlated with immunoparalysis [154]<br><br>Cardiac arrest: S100B↑ worse neurological outcome serum [150]<br><br>Acute myocardial infarction: Plasma S100A1↑ [155] | Cardiac arrest: plasma nDNA↑ [154]<br><br>Myocardial infarction: plasma mtDNA↑ [156] |

***Continuation Table 1***

sustain respiratory failure and, in severe cases, can spread systemically, resulting in multi organ dysfunction syndrome (MODS) [125]. Even protective ventilation strategies that do not cause direct mechanic tissue damage have been shown to elicit an inflammatory response and subsequent lung injury [126]. The mechanisms behind this so-called 'biotrauma' [127] are assumed to be related to the mechanical ventilation-induced release of DAMPs and the subsequent activation of the immune system. It is known that activation of TLR-4 plays an important role in the development of VILI [126], as does activation of NF-κB [128]. Furthermore, synergistic detrimental effects of a primary insult, for instance bacterial sepsis, and mechanical ventilation has been demonstrated multiple times [129-131]. Moreover, the direct involvement of several DAMPs in VILI have been demonstrated, as outlined below. In patients, HMGB1 level in bronchoalveolar lavage fluid (BALF) increases in response to mechanical ventilation [132, 133]. Furthermore, when administered intratracheally in mice, HMGB1 exerts inflammatory effects, while neutralization results in less inflammation and lung injury [133, 134]. HSPs also appear to play a role in the pathogenesis of VILI. For example, increased level of extracellular HSP72 in BALF and plasma of acute lung injury patients has been reported [135]. Furthermore, murine studies have revealed that HSP72 exerts its inflammatory effects via TLR-4 [136]. Concerning the S100 protein family, both S100A9 and S100A12 (EN-RAGE) are increased in BALF of patients with ARDS compared with healthy controls [137, 138], although no studies have been undertaken to investigate the effects of neutralization of HSPs or S100 proteins in VILI. Concerning nucleic acids, previous studies have shown that acid aspiration results in profoundly increased mtDNA level in BALF of mice [139], and that exogenous administration of mtDNA in the lungs or circulation of rats and mice elicits inflammatory lung injury in a TLR9-dependent manner [140, 141]. However, the effect of endogenous mtDNA release or neutralization during mechanical ventilation and its role in ventilator-induced inflammation has not yet been investigated. Low molecular weight (LMW) hyaluronan was also found to be increased in BALF of patients with acute respiratory distress syndrome (ARDS) [142]. Moreover, inhibiting synthesis of LMW hyaluronan in septic ventilated mice resulted in reduced lung injury [143], suggesting possible therapeutic potential in ventilated critically ill patients.

**Cardiac arrest**

Survival after cardiac arrest is low, even when return of spontaneous circulation is achieved [144-147]. In part, this is due to the development of the post-cardiac arrest syndrome, a condition demonstrating similarities to severe sepsis [148, 149]. DAMPs are assumed to play an important role in the pathogenesis of this complication.

Both HMGB1 and S100B concentrations in spinal fluid and serum are increased in patients following cardiac arrest, and are associated with poor neurological outcome [150, 151]. In contrast to these observation, several animal studies have demonstrated that HMGB1 promotes regeneration and recovery if injected into the heart after myocardial infarction [152, 153]. Plasma levels of S100A12, HSP70, nDNA, and mtDNA are also increased following cardiac arrest and out-of-hospital resuscitation, and especially HSP70 level is associated with the development of immunoparalysis [154]. Increased plasma levels of S100A1, HSP70, and mtDNA have also been reported after myocardial infarction [155-157], and mtDNA level correlated with the development of immunoparalysis [156]. To date, apart from the abovementioned studies on intracardial HMGB1 injections, treatment options targeting DAMPs in cardiac arrest have not been studied.

In conclusion, a wide range of DAMPs are implicated in disease states frequently encountered in critically ill patients. Nevertheless, research on therapeutic interventions targeting DAMPs is in its infancy, especially in humans, and faces challenges in discriminating between beneficial and harmful effects of DAMPs, as the relationship between DAMP release and outcome is not always unambiguous [36, 104, 119, 123]. Before progress can be made in this respect, increased knowledge is warranted on the multiple functions of and interactions between different DAMPs in critical illness. Along these lines, as many different DAMPs binding to different pattern recognition receptors can be released simultaneously, increased knowledge on interactions between these signaling receptors is required as well. Next to therapeutic targets, DAMPs may represent suitable biomarkers in intensive care-related conditions. For example, in sepsis, a marker that discriminates between the bacterial phase and the sterile SIRS phase is highly warranted to determine nature and timing of treatment strategies in those patients [8]. Nuclear DNA and mtDNA concentrations in plasma may represent a promising marker for this purpose [94]. In trauma patients, S100B protein appears to be the most valuable marker for the differential diagnosis in traumatic brain injury [36, 120, 158]. Other biomarkers, that are immunologically inactive, in contrary to DAMPs, could also be of value in this respect.
Moreover, caution is warranted in translating the results obtained in animal models, such as those described in Table 2, to patients in the ICU. Major differences exist between the immune system of animals and humans, and a clinical setting is often not comparable to a standardized setting used in animal models. This has contributed to disappointing results of many clinical trials in the past. For instance, although a wide variety of anti-inflammatory therapies showed very promising

| | | HMGB1 | HSP |
|---|---|---|---|
| **Sepsis** | ***E.coli*/CLP** | CLP (mice): anti-HMGB1: survival↑. HMGB1 administration: mortality↑ [13] | CLP (rats): Glutamin-induced enhanced HSP expression: lung tissue metabolic function↑, mortality↓.<br>Additional inhibition of HSPs by quercetin: no survival effect of glutamine [102] |
| | **Endotoxemia** | | Lethal endotoxemia (mice): inhibition of HSPs by quercetin: mortality, Plasma TNF-α and IL-1β↓ [101] |
| **Trauma** | | Bilateral femur fracture (mice): anti-HMGB1: inflammation↓, no effects in TLR4-deficient mice [112]<br><br>Hemorrhage (30% blood loss, mice): anti-HMGB1 administration: NFκB+cytokines in lung↓, lung accumulation of neutrophils↓ [114]<br><br>Hemorrhagic shock (MAP 25-30 mmHg, mice): anti-HMGB1: survival↑, bacterial translocation from gut↓, plasma IL-6+IL-10↓ [113]<br><br>Liver I/R (mice): anti-HMGB1: liver damage ↓, no effects in TLR4-deficient mice. HMGB1 administration: liver damage↑ [116] | Not investigated. |

***Table 2 – Animal intervention studies on DAMPs in intensive care-related conditions***

*I/R=ischemia/reperfusion; CLP=cecal ligation and puncture; MAP=mean arterial pressure*

| S100 proteins | Nucleic acids |
|---|---|
| *E.coli* abdominal sepsis (mice): S100A8/A9 knockout mice protected [41]<br><br>*E.coli* abdominal sepsis (mice): S100A9 deficient mice exhibit improved defense and decreased systemic inflammation [103] | CLP (mice): DNase treatment: coagulation+inflammation↓, organ damage↓, survival↑ [161] |
| Lethal endotoxemia: S100A8/A9 knockout mice protected [41] | |
| Intraneural injection of S100A8/A9 into the sciatic nerve (rats): myeloid cell migration into the nerve [118] | Not investigated. |

***Continuation Table 2***

| | HMGB1 | HSP |
|---|---|---|
| **VILI** | 4h mechanical ventilation (30 ml/kg, rabbits): anti-HMGB1: oxygenation↑, microvascular permeability, neutrophil influx, TNF-α in bronchoalveolar lavage fluid (BALF)↓ [133]<br><br>Endotoxin-induced lung injury (mice): anti-HMGB1: neutrophil influx, pulmonary edema↓, no effect on pulmonary IL-1β, TNF-α, MIP-2 [134] | Inhalation of Hsp72 (mice): bronchoalveolar lavage fluid (BALF) KC, TNF-α, neutrophil recruitment ↑. No effect in TLR4-deficient mice [136] |
| **Cardiac arrest** | Myocardial infarction (mice), HMGB1 administration: recovery of cardiac performance↑ [152, 153] | Not investigated. |

***Continuation Table 2***

results in preclinical sepsis models, none of these have eventually proved to be of benefit in actual septic patients [159, 160].

Taken together, although additional studies are warranted to confirm applicability in clinical practice, research into DAMPs is a promising field to develop tools that can eventually have an impact on care, especially of critically ill patients.

## Outline of this thesis

The first part of this thesis is entitled "DAMPs in critically ill patients" and comprises studies on DAMPs, immune responses, and the relationship between these two in several ICU-related diseases. In chapter 2, the role of DAMPs in the impaired immune response observed in a large cohort of trauma patients is investigated. A similar impaired immune response, which is related to DAMP release, is studied in cardiac arrest patients, as described in chapter 3. In chapter 4, DAMP release induced by chemotherapy and its effects on impaired immune responses in patients with acute leukemia is evaluated. In septic shock patients, the mechanisms

| S100 proteins | Nucleic acids |
|---|---|
| Not investigated. | Gastric acid aspiration (mice): mtDNA in bronchoalveolar lavage fluid (BALF) ↑ [139]<br><br>Intratracheal administration of mtDNA (mice): pulmonary inflammation↑, effects abrogated by TLR9-inhibitor or siRNA [141]<br><br>Intratracheal administration of mtDNA (rats), lung inflammation and injury↑, TLR9 expression↑, nDNA: no effects [140] |
| Acute myocardial infarction (mice): intracardiac S100A1 injection: normalization of ICAM1 and collagen. Anti-S100A1: infarct size↑, left ventricular functional performance post-MI↓ [155] | Not investigated. |

***Continuation Table 2***

(markers of inflammation, shock, and organ damage) through which the DAMPs mtDNA and nDNA have an impact on mortality were studied, the results of which are presented in chapter 5. As outlined above, different DAMPs can trigger various PRRs simultaneously. In chapter 6, the interactions between those receptors and the extent to which these interaction pathways are evolutionary conserved are investigated.

The second part of this thesis entitled "DAMPs and ventilator-induced inflammation" focuses on the sterile inflammatory response induced by mechanical ventilation, and the role of DAMPs in this process. Chapter 7 concerns the role of mtDNA and its receptor TLR9 in ventilator-induced inflammation in mice. Chapter 8 comprises a murine study on the immunological mechanisms of mechanical ventilation-induced inflammation, proposing a mechanism for activation of the pro-inflammatory cytokine IL-1β in this process.

Next to the DAMPs described in this thesis, several other immunologically inactive molecules could serve as biomarkers to predict outcome or complications in the trauma population we have studied in this thesis. In part three of this thesis,

entitled “Biomarkers in trauma”, two of these markers are investigated. Chapter 9 describes the temporal relationship between trauma and intestinal damage during the first days of hospital admission using the biomarker intestinal fatty acid binding protein (iFABP), and the factors that play a role in the development of intestinal damage in these patients. In chapter 10, results on the predictive value of soluble urokinase plasminogen activator receptor (suPAR, an inflammation-associated marker frequently described in several ICU populations) for mortality in trauma patients is presented.
This thesis is concluded by a general discussion and future perspectives of the findings presented in this thesis and a summary.

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# Part I

## DAMPs in critically ill patients

# Chapter 2

## Plasma levels of Danger-Associated Molecular Patterns are associated with immune suppression in trauma patients

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## Abstract

**Purpose** Danger-associated molecular patterns (DAMPs) released following trauma could contribute to an immune suppressed state that renders patients vulnerable towards nosocomial infections. We investigated DAMP release in trauma patients, starting in the pre-hospital phase, and assessed its relationship with immune suppression and nosocomial infections.

**Methods** Blood was obtained from 166 adult trauma patients at the trauma scene, ER, and serially afterwards. Circulating levels of DAMPs and cytokines were determined. Immune suppression was investigated by determination of HLA-DRA gene expression and *ex vivo* LPS-stimulated cytokine production.

**Results** Compared with healthy controls, plasma levels of nuclear DNA (nDNA) and heat shock protein-70 (HSP70), but not mitochondrial DNA were profoundly increased immediately following trauma, and remained elevated for 10 days. Plasma cytokines were increased at the ER, and levels of anti-inflammatory IL-10, but not of pro-inflammatory cytokines peaked at this early time-point. HLA-DRA expression was attenuated directly after trauma, and did not recover during the follow-up period. Plasma nDNA ($r=-0.24$, $p=0.006$) and HSP70 ($r=-0.38$, $p<0.0001$) levels correlated negatively with HLA-DRA expression. *Ex vivo* cytokine production revealed an anti-inflammatory phenotype already at the trauma scene which persisted in the following days, characterized by attenuated TNF-α and IL-6, and increased IL-10 production. Finally, higher concentrations of nDNA and a further decrease of HLA-DRA expression were associated with infections.

**Conclusions** Plasma levels of DAMPs are associated with immune suppression, which is apparent within minutes/hours following trauma. Furthermore, aggravated immune suppression during the initial phase following trauma is associated with increased susceptibility towards infections.

## Introduction

The survival of multiple trauma patients has improved significantly during the past decades [1]. However, despite improvements in both traffic safety and pre- and in-hospital management, severe trauma remains a main cause of death among young people worldwide [2]. In 2014, 25,845 people were killed and over 203,500 seriously injured in road accidents in the EU alone [3]. Roughly, trauma-related mortality can be divided in two categories. Early deaths are mainly attributed to neurological damage or severe blood loss directly related to the trauma. The patients that survive the initial trauma often develop nosocomial infections or sepsis [4], representing a significant cause of late mortality in trauma patients. The increased susceptibility of trauma patients to develop infections is mediated by a suppressed state of the immune system that develops after trauma [4-9]. Two frequently used hallmarks of the immune suppressed state after trauma are attenuated production of cytokines by leukocytes *ex vivo* stimulated with Pathogen Associated Molecular Patterns (PAMPs) such as LPS, and decreased leukocyte HLA-DR expression [6, 8, 10-13].

Release of Danger Associated Molecular Patterns (DAMPs), which can elicit an immune response very similar to the response to PAMPs from invading pathogens in sepsis [14, 15], could contribute to immune suppression in trauma patients. DAMPs can both be actively released by ischaemic cells as danger signals or originate from damaged or dead cells as debris [16, 17]. An example of a DAMP that can be released in case of cell damage is mitochondrial DNA (mtDNA), which can trigger an immune response via Toll-like receptor 9 [18, 19]. Moreover, heat shock protein (HSP)-70 is released following trauma [20], and has been shown to induce immune cell deactivation [21]. Furthermore, previous studies have indicated that free nuclear DNA (nDNA) in plasma is a marker for cell damage or death, because it is one of the many cell components released if a cell is ruptured [19, 22]. Therefore, it might be an indicator of general DAMP release. However, the role of these DAMPs in the immune response after trauma and the possible development of a suppressed state of the immune system is unknown.

Taken together, although immune suppression and nosocomial infections are frequently described phenomena in trauma patients, the role of DAMPs that trigger pro- and anti-inflammatory responses remains elusive. The aim of this study was to investigate the release of DAMPs following trauma, starting in the very early (pre-hospital) phase, and to assess its relationship with immune suppression and nosocomial infections.

## Methods

### Study population

Adult trauma patients (n=166) admitted to the trauma care unit at the ER of the Radboud University Nijmegen Medical Centre were included in the study. Exclusion criteria were expected risks of blood sampling at the trauma scene (e.g. jeopardizing the clinical handling of the patient), known HIV/AIDS, known malignancies, and use of steroids (all dosages and types of administration) or other immunomodulatory medication previously to the trauma. SDD was administered to all patients who were admitted to the ICU (n=101), as part of standard ICU protocol. Therefore, comparisons between ICU patients who did and did not receive antibiotics could not be made. Of the patients who were not admitted to the ICU (n=65), only 7 received (prophylactic) antibiotics; this group size does not allow for statistical analysis. Furthermore, comparing patients that did not receive antibiotics (and thus by definition were not admitted to the ICU) with patients that did receive antibiotics (all ICU patients and the 7 non-ICU patients that received antibiotics) does not yield meaningful information, because of major differences in trauma/disease severity, placement of catheters, intubation etc.

The study was carried out in the Netherlands in accordance with the applicable rules concerning the review of research ethics committees and informed consent (CMO2011/380, NL38169.091.11). All patients or legal representatives were informed about the study details at the first opportunity, usually within 1 day after admission. The local ethical committee that approved the study protocol agreed that it was not possible to do this at an earlier stage. Written informed consent was obtained from the patient or his/her legal representative if vena puncture was necessary to obtain blood samples. All determinations and data handing were performed under the guidelines of The National Institutes of Health and in accordance with the declaration of Helsinki and its later amendments.

Control samples (n=12) were obtained from healthy male volunteers (median age 22 [range 19-27]) participating in an experimental human endotoxemia trial (CMO2012/455, NCT01835457). Samples were obtained from the control group at baseline, before administration of endotoxin. Written informed consent was obtained from all of these volunteers prior to screening and inclusion in the study.

### Sample and data collection

Blood was sampled shortly after trauma at the trauma scene by the Helicopter Emergency Medical Services (HEMS) before hospital admission ('pre-hosp') if applicable, at arrival at the Emergency Room (ER), and at day 1, 3, 5, 7 and 10

following trauma. The HEMS response time (time between notification of the HEMS team and arrival at the trauma scene) was 16 [12-19] minutes. Time spent at the trauma scene by the HEMS team was 23 [15-29] minutes and the interval between sampling at the trauma scene (time-point pre-hosp) and sampling at the ER was 39 [33-45] minutes.

Lithium Heparin (LH) anti-coagulated blood was obtained for *ex vivo* stimulation experiments as described below, which were performed immediately after sampling. Ethylenediaminetetraacetic acid (EDTA) and LH anti-coagulated blood was centrifuged after withdrawal at 1,600xg at 4$^0$C for 10 minutes, after which plasma was stored at -80$^0$C until further analysis. EDTA plasma for real time quantitative PCR (qPCR) analysis was centrifuged again at 16,000xg at 4$^0$C for 10 minutes to remove potential remaining cells and cell debris. The supernatant was stored at -80$^0$C until further analysis. Blood for mRNA analysis was sampled in PAXgene blood RNA tubes (Qiagen, Valencia, CA, USA) and stored according to the manufacturer's instructions.

Clinical parameters and demographic data were obtained from electronic patient files. Injury Severity Scores (ISS) were supplied by the Regional Emergency Healthcare Network. Infection within 28 days was defined as the presence of fever and/or other infectious symptoms (pain, swelling, erythema) with leukocytosis and positive cultures and/or another visible or otherwise proven infection focus corresponding with the symptoms of the patient. The attending physicians were blind to the immune investigation results as these assays were performed after collection of all samples from each patient.

**Plasma DAMP levels**

Plasma from double-centrifuged EDTA anti-coagulated blood was diluted 1:1 with phosphate buffered saline solution (PBS) after which DNA was isolated using the QIAamp DNA Blood Midi Kit (Qiagen, Valencia, CA, USA), using the 'Spin Protocol' as described by the manufacturer. Isolated DNA was stored at -20$^0$C until further analysis. qPCR was performed using iQ SYBR Green PCR Master Mix (Bio-Rad Laboratories, Hercules, CA, USA) on a CFX96 Real-Time PCR Detection system (Bio-Rad Laboratories, Hercules, CA, USA). A primer pair specific for the GAPDH gene present in all nucleated cells of the body was used for quantification of nuclear (n)DNA levels: forward 5'-AGCACCCCTGGCCAAGGTCA-3', reverse 5-CGGCAGGGAGGAGCCAGTCT-3', For quantification of mitochondrial (mt)DNA levels, the following primer pair specific for the mitochondrially encoded NADH dehydrogenase 1 (MT-ND1) gene was used: forward 5'-GCCCCAACGTTGTAGGCCCC-3' and reverse 5'AGCTAAGGTCGGGGCGGTGA-3'.

Primer pairs were obtained from Biolegio (Nijmegen, the Netherlands). Samples were analyzed in duplicate and DNA isolated from blood obtained from a healthy volunteer was used on each plate as a calibrator (CV% of 1.48% [GAPDH] and 0.41% [mtDNA] between plates). Plasma nDNA and mtDNA levels are expressed as fold-change relative to the calibrator sample using the formula 2^ΔCt.
Plasma concentrations of HSP70/HSPA1A were determined batchwise using ELISA according to the manufacturer's instructions (R&D systems, Minneapolis, MN, USA).

**Plasma cytokine concentrations**
Plasma concentrations of pro-inflammatory cytokines Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-6, and IL-8, and the anti-inflammatory cytokine IL-10 were analyzed batchwise in plasma obtained from EDTA anti-coagulated blood using a simultaneous Luminex assay according to the manufacturer's instructions (Milliplex; Millipore, Billerica, MA, USA).

***Ex vivo* cytokine production**
Leukocyte cytokine production capacity was determined by challenging whole blood from the patients with LPS *ex vivo* using an in-house developed system with pre-filled tubes described in detail elsewhere [25]. Briefly, 0.5 mL of blood was added to tubes pre-filled with 2 mL culture medium as negative control or 2 mL culture medium supplemented with 12.5 ng/mL *Escherichia coli* lipopolysaccharide (LPS, serotype O55:B5 [Sigma Aldrich, St Louis, MO, USA], end concentration: 10 ng/mL). Cultures were incubated at 37°C for 24 hours, centrifuged, and supernatants were stored at -80 C until analysis. Concentrations of TNF-α, IL-6, and IL-10 were determined batchwise by ELISA, according to the manufacturer's instructions (R&D systems, Minneapolis, MN, USA). *Ex vivo* cytokine production data were censored at time of infection diagnosis, because infections can induce immune alterations.

**HLA-DRA mRNA expression**
RNA was isolated from blood collected in Paxgene blood RNA tubes (Qiagen, Valencia, CA, USA). cDNA was synthesized from total RNA using the iScript cDNA Synthesis Kit (Bio-rad, Hercules, CA, USA). Subsequent qPCR analysis was performed using TaqMan gene expression assays (Life Technologies, Paisley, UK) for the reference gene peptidylpropylisomeras B (PPIB) (#Hs00168719_m1) and HLA-DRA (#Hs00219575_m1) on a CFX96 Real-Time PCR Detection system (Bio-Rad, Hercules, CA, USA). We chose PPIB based on its stability in inflammatory

conditions in peripheral whole blood [26] and previous use as a reference gene for HLA-DRA [27]. We chose the HLA-DRA gene because it was shown to correlate well with flow cytometric analysis of mHLA-DR [27-29], an established marker of immune suppression. HLA-DRA expression levels are expressed as fold-change relative to the expression of PPIB in the same sample using the formula 2^ΔCt. HLA-DRA data were censored at time of infection diagnosis, because infections can decrease HLA-DR expression.

**Statistical analysis**

Data presented in tables and text are expressed as median [interquartile range] and data in figures as geometric mean±95% CI. Mann-Whitney U and Fischer exact tests were used to investigate differences between two groups as appropriate. Differences between patient data at the various time-points and data of healthy controls were performed using Kruskal-Wallis with Dunn's post-hoc tests. Differences between time-to-infection curves were tested using Log-rank (Mantel-Cox) tests. A Cox proportional hazard model was used to adjust the relationship between HLA-DRA expression and time-to-infection for the usual clinical confounders age and ISS [10]. Correlations were calculated using Spearman correlation. All analyses were performed with available data of the corresponding time-points. Due to missing values at certain time-points or patients that were lost to follow-up, patient numbers in the analyses vary. Principal component analysis was performed to explore the expected covariation between multiple laboratory variables and their relationship with injury severity, thereby preventing the need to list all individual correlations [30]. No imputation was used, as missing values were judged to be non-random, i.e. blood for [X] was sampled at day [Y] and could therefore not be obtained in patients who died early. Instead, a core dataset of variables and patients without missing values was established. Measurements were log-transformed, mean-substracted and z-score was calculated on which principal component analysis was performed based on the singular value distribution in a Python script. All other statistical analyses were performed using SPSS statistics version 22 (IBM Corporation, Armonk, NY, USA) and Graphpad Prism version 5 (Graphpad Software, La Jolla, USA). A p value of $<0.05$ was considered statistically significant.

## Results

### Patient characteristics

A total of 166 patients were included between August 2010 and May 2013, of which the characteristics are listed in Table 1. The majority of patients suffered from head/neck or chest injury.

| | **Total (n=166)** |
|---|---|
| Gender | Male: n=123 (74%)<br>Female: n=43 (26%) |
| Age (years) | 50 [31-67] |
| Injury Severity Score | 26 [17-37] |
| Head/Neck injury (ISS region 1) | n=129, 78% |
| Face injury (ISS region 2) | n=47, 28% |
| Chest injury (ISS region 3) | n=96, 58% |
| Abdomen or pelvic contents injury (ISS region 4) | n=44, 27% |
| Extremities or pelvic girdle injury (ISS region 5) | n=78, 47% |
| External injury (ISS region 6) | n=77, 46% |
| ICU admission * | n=101 (61%) |
| Mechanical ventilation * | n=96 (95%) |
| Vasopressor therapy * | n=35 (35%) |
| ICU length of stay (days) | 3 [1-7] |
| Corticosteroids administered * | n=8 (5%) |
| Transfusion of blood products* | n=24 (14%) |
| Hospital length of stay (days) | 8 [1-17] |
| 28-day survival | n=127 (77%) |
| 28-day survival (among initial trauma survivors) | n=127/147 (86%) |

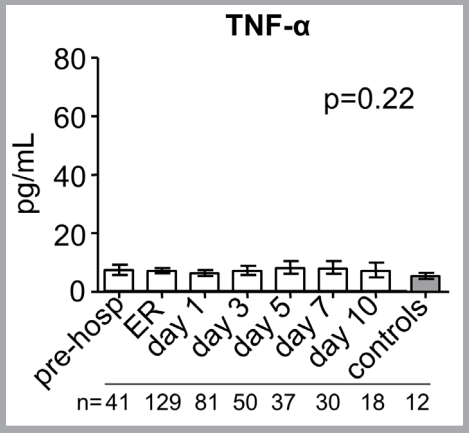
***Table 1 – Patient characteristics*** *Initial trauma survivors were defined as patients who survived the initial 7 days following trauma. Vasopressor therapy always consisted of noradrenaline. Corticosteroids included all types of administration. Blood products included erythrocytes, thrombocytes, and fresh frozen plasma. * admitted to, or used within 28 days after hospital admission.*

**Plasma DAMPs**

Plasma nDNA levels were profoundly increased at time-points pre-hospital and ER compared with healthy controls (Figure 1A). Although levels decreased in the later phase, they remained elevated during the entire follow-up period. Plasma mtDNA levels were also elevated in trauma patients compared with healthy controls (Figure 1B), although this increase was not as pronounced and only reached statistical significance at two time-points. Similar to nDNA, plasma HSP70 concentrations in trauma patients were highest shortly after trauma and decreased later on, but nevertheless remained elevated compared with healthy controls at all time-points (Figure 1C).

**Plasma cytokines**

Plasma TNF-α concentrations in trauma patients were not elevated at any time-point compared with levels found in healthy controls and did not change over time (supplementary Figure 1). Plasma IL-6 levels were elevated from time-point ER until day 7 post-trauma (Figure 1D), while IL-8 levels were slightly, but significantly increased compared with healthy controls from time-point ER and remained elevated during the entire follow-up period (Figure 1E). Both cytokines showed highest levels at day 1. Plasma IL-10 concentrations in trauma patients showed a distinct peak at the ER, and remained significantly higher compared with healthy controls until day 1 (Figure 1F). Plasma nDNA levels measured at the ER correlated with plasma IL-8 ($r=0.40$, $p<0.0001$, $n=121$), IL-6 ($r=0.47$, $p<0.0001$, $n=121$), and IL-10 ($r=0.45$, $p<0.0001$, $n=121$) concentrations at the same time-point. Plasma HSP70 levels at the ER correlated with plasma IL-8 ($r=0.40$, $p<0.0001$, $n=100$), IL-6 ($r=0.45$, $p<0.0001$, $n=100$), and IL-10 ($r=0.48$, $p<0.0001$, $n=100$) levels at that time-point.




***Supplementary Figure 1 – Plasma TNF-α levels*** *Plasma levels of TNF-α in trauma patients and healthy controls. The number of patients/controls included at each time-point is indicated below each time-point. The number of patients/controls included at each time-point is indicated below each time-point. * indicates $p<0.05$ compared with healthy controls. pre-hosp = pre-hospitally, ER = Emergency room.*

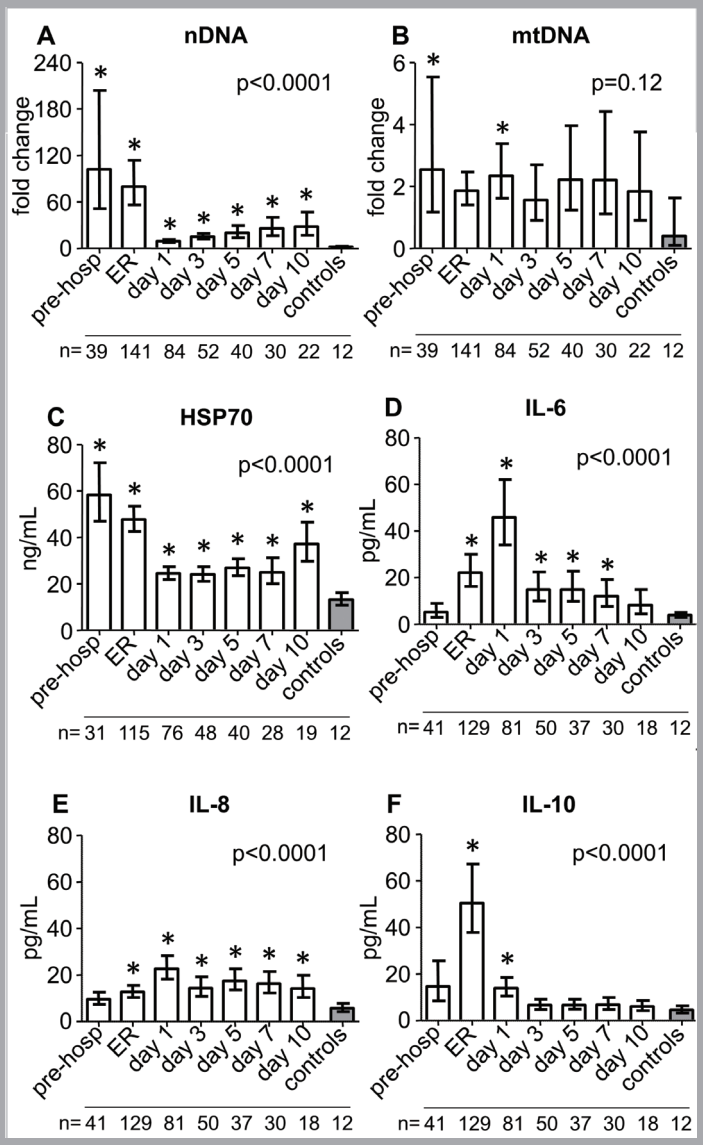



***Figure 1 – Plasma DAMP and cytokine levels*** *Plasma levels of nDNA (nuclear DNA, indicator of general DAMP release, panel A), mtDNA (mitochondrial DNA, DAMP, panel B), HSP70 (DAMP, panel C), and cytokines (panels D-F) in trauma patients and healthy controls. The number of patients/controls included at each time-point is indicated below each time-point. The number of patients/controls included at each time-point is indicated below each time-point. * indicates p<0.05 compared with healthy controls. pre-hosp = pre-hospitally, ER = Emergency room.*

**Immune suppressed state**

HLA-DRA mRNA expression in trauma patients was profoundly suppressed at all time-points compared with healthy controls (Figure 2A). Plasma nDNA and HSP70 levels negatively correlated with HLA-DRA expression at time-point ER (r=-0.24, p=0.006, n=130, and r=-0.38, p<0.0001, n=106, respectively), while plasma mtDNA levels did not correlate with HLA-DRA expression (r=-0.09, p=0.33, n=130). *Ex vivo* cytokine production capacity was investigated in a subgroup of patients (n=36), whose characteristics were comparable to the entire patient cohort (supplementary Table 1). The capacity of leukocytes to produce pro-inflammatory cytokines TNF-α and especially IL-6 upon *ex vivo* stimulation with LPS was severely suppressed at the trauma scene and during the first days of hospital admission compared with healthy controls (Figure 2, panels B and C). In sharp contrast, *ex vivo* production of the anti-inflammatory cytokine IL-10 was augmented in the first days after trauma compared with healthy controls (Figure 2D). This effect remained evident during the entire 10-day follow-up period (data not shown). *Ex vivo* TNF-α and IL-6 production at the ER correlated positively with HLA-DRA expression (r=0.43, p=0.02, n=30, and r=0.58, p=0.001, n=30, respectively). This was not the case for *ex vivo* IL-10 production (r=0.22, p=0.25, n=30).

**Relationship between injury severity, DAMPs, cytokines, and HLA-DRA**

To comprehensively investigate the relationship between injury severity and the mediators measured, we performed principal component analysis on data of nDNA, mtDNA, HSP70, IL-10, IL-6, IL-8, TNF-α, and HLA-DRA expression at the ER. In concordance with the individual correlations shown, the first principal component (PC1) had high loadings in the same direction of plasma nDNA, HSP70, IL-10, IL-6, IL-8, and TNF-α levels, while HLA-DRA had a smaller negative loading. PC1 had a total explained variance of 46% and correlated with ISS (r=0.64 p<0.0001, supplementary Figure 2).

**Susceptibility towards infections**

Thirty-three patients (20%) developed an infection during the first 28 days following trauma (characteristics of infected and non-infected patients provided in supplementary Table 2, part I). Time until infection was 7 [4-12] days. Types of infection were pneumonia (n=21), wound infection (n=5), meningitis (n=4), urinary tract infection (n=2), central line infection (n=1), empyema (n=1), bacteremia eci (n=1), paronychia (n=1), and unknown (n=1). Four patients suffered from two infections. Eighteen out of 21 patients with pneumonia were intubated at hospital admission and 15 at the moment of pneumonia diagnosis. Gender,

| | **Total (n=36)** |
|---|---|
| Gender | Male: n=31 (86%)<br>Female: n=5 (14%) |
| Age (years) | 51 [29-60] |
| Injury Severity Score | 26 [18-41] |
| Head/Neck injury (ISS region 1) | n=31, 86% |
| Face injury (ISS region 2) | n=10, 28% |
| Chest injury (ISS region 3) | n=19, 53% |
| Abdomen or pelvic contents injury (ISS region 4) | n=7, 19% |
| Extremities or pelvic girdle injury (ISS region 5) | n=12, 33% |
| External injury (ISS region 6) | n=15, 42% |
| ICU admission * | n=25 (69%) |
| Mechanical ventilation * | n=23 (92%) |
| Vasopressor therapy * | n=16 (64%) |
| ICU length of stay (days) | 3 [1-9] |
| Corticosteroids administered * | n=1 (3%) |
| Transfusion of blood products* | n=6 (17%) |
| Hospital length of stay (days) | 6 [1-12] |
| 28-day survival | n=25 (69%) |

***Supplementary Table 1 - Characteristics of patients in which ex vivo cytokine production was investigated*** *Vasopressor therapy always consisted of noradrenaline. Corticosteroids included all types of administration. Blood products included erythrocytes, thrombocytes, and fresh frozen plasma. * admitted to, or used within 28 days after hospital admission.* admitted to, or used within 28 days after hospital admission.*

age, injury severity and injury location were comparable between patients who developed an infection within 28 days and those who did not. However, patients who developed an infection following trauma were more frequently admitted to the ICU, received more transfusions, and required a longer length of stay, both at the ICU and in the hospital. Furthermore, vasopressor therapy and corticosteroid use tended to be higher in patients who developed an infection. 28-Day survival was higher in patients who developed an infection compared with those who did not, likely due to direct trauma-related deaths. Indeed, when analyzing the data of patients who survived the initial phase after trauma, no difference in 28-day survival was observed (supplementary Table 2, part II). ISS was slightly higher in patients who survived the initial phase after trauma and developed an infection,

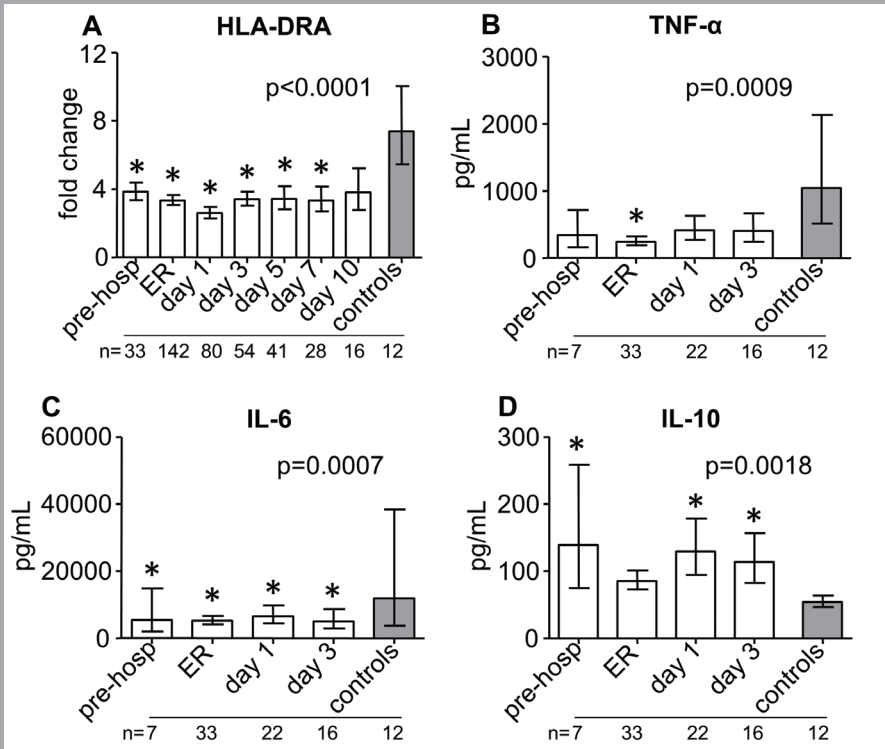



***Figure 2 – Markers of immune suppression*** *Leukocyte HLA-DRA mRNA expression in trauma patients and healthy controls, expressed as fold change compared with PPIB (panel A). Cytokine concentrations in supernatants after 24 hours of whole-blood LPS stimulation in patients during the first 3 days after trauma and in healthy controls (panels B, C, and D). The number of patients/controls included at each time-point is indicated below each time-point. * indicates p<0.05 compared with healthy controls. pre-hosp = pre-hospitally, ER = Emergency room.*

and these patients were more frequently admitted to the ICU. Furthermore, vasopressor therapy, transfusions and corticosteroids were more frequently used, and ICU and hospital length of stay were increased in these patients.

Plasma mtDNA and nDNA levels at ER were higher in patients who developed an infection within 28 days compared with patients who did not (2.5 [1.4-6.6] vs. 1.4 [0.5-4.0] fold change, p=0.046 for mtDNA, and 265.7 [30.7-1131.3] vs. 61.9 [11.7-367.5] fold change, p=0.02 for nDNA). For HSP70, no differences were observed between these two groups (57.4 [26.0-79.8] vs. 47.2 [33.9-72.0] ng/mL, p=0.89).

Previous studies indicate that the change in HLA-DR expression over time better

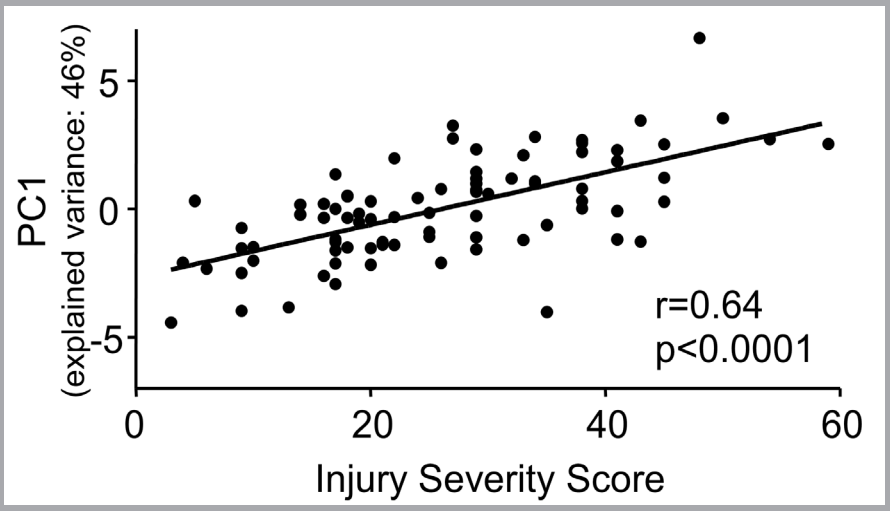
| All patients (n=166) | | | |
|---|---|---|---|
| | **Infection (n=33, 20%)** | **No infection (n=133, 80%)** | **P value** |
| Gender | Male: n=26 (79%)<br>Female: n=7 (21%) | Male: n=97 (73%)<br>Female: n=36 (27%) | 0.66 |
| Age (years) | 48 [28-61] | 50 [32-67] | 0.54 |
| Injury Severity Score | 29 [20-38] | 25 [17-36] | 0.16 |
| Head/Neck injury (ISS region 1) | n=29, 88% | n=100, 75% | 0.16 |
| Face injury (ISS region 2) | n=10, 30% | n=37, 28% | 0.83 |
| Chest injury (ISS region 3) | n=21, 64% | n=75, 56% | 0.56 |
| Abdomen or pelvic contents injury (ISS region 4) | n=11, 33% | n=33, 25% | 0.38 |
| Extremities or pelvic girdle injury (ISS region 5) | n=19, 58% | n=59, 44% | 0.18 |
| External injury (ISS region 6) | n=16, 49% | n=61, 46% | 0.85 |
| ICU admission | n=28 (85%) | n=73 (55%) | **0.001** |
| Mechanical ventilation during ICU stay | n=28 (100%) | n=68 (93%) | 0.32 |
| Vasopressor therapy * | n=14 (50%) | n=21 (29%) | 0.06 |
| ICU length of stay (days) | 11 [5-20] | 2 [1-6] | **<0.0001** |
| Corticosteroids administered * | n=4 (12%) | n=4 (3%) | 0.051 |
| Transfusion of blood products* | n=9 (27%) | n=15 (11%) | **0.03** |
| Hospital length of stay (days) | 30 [17-38] | 5 [1-11] | **<0.0001** |
| 28-day survival | n=30 (91%) | n=97 (73%) | **0.04** |

***Supplementary Table 2 - Patient characteristics according to the development of infections within 28 days. Part I.***

| Initial trauma survivors (n=147) | | | |
|---|---|---|---|
| | **Infection (n=33, 22%)** | **No infection (n=114, 78%)** | **P value** |
| Gender | Male: n=26 (79%)<br>Female: n=7 (21%) | Male: n=84 (74%)<br>Female: n=30 (26%) | |
| Age (years) | 48 [28-61] | 49 [31-65] | 0.77 |
| Injury Severity Score | 29 [20-38] | 23 [14-34] | **0.048** |
| Head/Neck injury (ISS region 1) | n=29, 88% | n=81, 71% | 0.07 |
| Face injury (ISS region 2) | n=10, 30% | n=30, 26% | 0.66 |
| Chest injury (ISS region 3) | n=21, 64% | n=62, 54% | 0.43 |
| Abdomen or pelvic contents injury (ISS region 4) | n=11, 33% | n=27, 24% | 0.27 |
| Extremities or pelvic girdle injury (ISS region 5) | n=19, 58% | n=54, 47% | 0.33 |
| External injury (ISS region 6) | n=16, 49% | n=53, 47% | 0.85 |
| ICU admission | n=28 (85%) | n=54 (47%) | **<0.0001** |
| Mechanical ventilation | n=28 (100%) | n=50 (93%) | 0.29 |
| Vasopressor therapy * | n=14 (50%) | n=14 (26%) | **0.048** |
| ICU length of stay (days) | 11 [5-20] | 2 [1-7] | **<0.0001** |
| Corticosteroids administered * | n=4 (12%) | n=3 (3%) | **0.045** |
| Transfusion of blood products* | n=9 (27%) | n=13 (11%) | **0.048** |
| Hospital length of stay (days) | 30 [17-38] | 7 [1-14] | **<0.0001** |
| 28-day survival | n=30 (91%) | n=97 (85%) | 0.57 |

***Supplementary Table 2 - Patient characteristics according to the development of infections within 28 days. Part II.***

***Supplementary Table 2 - Patient characteristics according to the development of infections within 28 days.*** *Initial trauma survivors were defined as patients who survived the initial 7 days following trauma. Vasopressor therapy always consisted of noradrenaline. Corticosteroids included all types of administration. Blood products included erythrocytes, thrombocytes, and fresh frozen plasma. * admitted to, or used within 28 days after hospital admission.* admitted to, or used within 28 days after hospital admission. Data of patients who developed an infection in the upper and lower parts of the table are identical, because all these patients survived the initial 7 days after trauma.*




***Supplementary figure 2 – Correlation between injury severity and principal component 1*** *Correlation between Injury Severity Score and the first principal component (PC1), to which IL-10, IL-6, IL-8, TNF-α, nDNA, and HSP70 contributed more than the expected average.*

predicts outcome and/or development of infections than absolute values of HLA-DR [10, 29, 31]. Accordingly, we investigated the relationship between change in HLA-DRA expression (increase or decrease between ER and day 3) and infections in a subgroup of patients from our cohort for which HLA-DRA data was available on these time-points . Patients exhibiting a decrease in HLA-DRA expression (ratio < 1) more likely developed an infection compared with patients who showed an increase (ratio > 1, Figure 3). The relationship between a decrease in HLA-DRA expression and development of infection remained apparent after correcting for age and ISS (hazard ratio [95% CI] of 3.02 [1.02-8.93], p=0.046). Furthermore, ICU and hospital length of stay were increased in patients with decreasing HLA-

DRA expression, while other characteristics were not significantly different (supplementary Table 3).

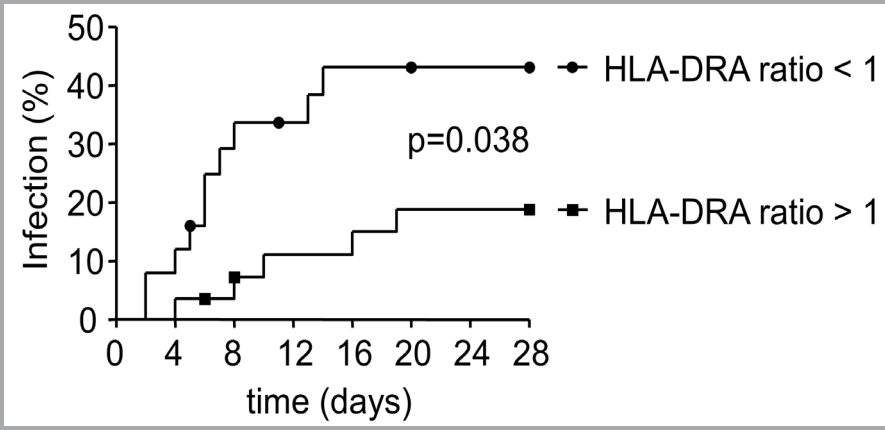



***Figure 3 – Relationship between change in HLA-DRA expression and development of infections during the first 28 days following trauma*** *Thirty-three (20%) patients developed an infection within 28 days following trauma. Patients exhibiting a decrease in HLA-DRA expression between ER and day 3 (ratio < 1) were more likely to develop an infection compared with those who showed an increase (ratio > 1). Symbols placed on lines indicate censored patients because of death.*

## Discussion

This study demonstrates that multi-trauma patients exhibit a suppressed state of the immune system already at the trauma scene, thus before admission of the patient to the hospital. This is characterized by low HLA-DRA expression and an anti-inflammatory cytokine pattern, both in- and ex vivo. Furthermore, we show that DAMPs are present in large quantities in the circulation during the pre-hospital phase and shortly after admission, and that DAMP levels are associated with the extent of immune suppression. Finally, our data demonstrate that further aggravation of immune suppression in the initial phase after trauma is associated with increased susceptibility towards infections. A conceptual representation of how DAMP release may lead to increased susceptibility towards infections following trauma is presented in Figure 4.

The pronounced general release of DAMPs, reflected by plasma nDNA levels, in the pre-hospital phase of trauma was associated with the immune suppression

| | HLA-DRA decrease (n=25, 47%) | HLA-DRA increase (n=28, 53%) | P value |
|---|---|---|---|
| Gender | Male: n=20 (80%)<br>Female: n=5 (20%) | Male: n=24 (86%)<br>Female: n=4 (14%) | 0.43 |
| Age (years) | 55 [33-64] | 54 [37-68] | 0.77 |
| Injury Severity Score | 29 [23-35] | 29 [19-37] | 0.80 |
| Head/Neck injury (ISS region 1) | n=18, 72% | n=25, 89% | 0.16 |
| Face injury (ISS region 2) | n=8, 32% | n=7, 25% | 0.76 |
| Chest injury (ISS region 3) | n=19, 76% | n=18, 64% | 0.39 |
| Abdomen or pelvic contents injury (ISS region 4) | n=10, 40% | n=8, 29% | 0.40 |
| Extremities or pelvic girdle injury (ISS region 5) | n=16, 64% | n=12, 43% | 0.17 |
| External injury (ISS region 6) | n=12, 48% | n=14, 50% | 1.00 |
| ICU admission | n=20 (80%) | n=22 (79%) | 1.00 |
| Mechanical ventilation during ICU stay | n=19 (95%) | n=20 (91%) | 0.76 |
| Vasopressor therapy * | n=10 (50%) | n=6 (27%) | 0.20 |
| ICU length of stay (days) | 11 [4-25] | 3 [1-7] | **<0.0001** |
| Corticosteroids administered * | n=4 (16%) | n=1 (4%) | 0.18 |
| Transfusion of blood products* | n=9 (36%) | n=4 (14%) | 0.11 |
| Hospital length of stay (days) | 21 [12-36] | 10 [7-21] | **0.02** |
| 28-day survival | n=19 (76%) | n=25 (89%) | 0.28 |

***Supplementary Table 3 - Patient characteristics according to change in HLA-DRA expression between ER and day 3*** *Vasopressor therapy always consisted of noradrenaline. Corticosteroids included all types of administration. Blood products included erythrocytes, thrombocytes, and fresh frozen plasma. * admitted to, or used within 28 days after hospital admission.* admitted to, or used within 28 days after hospital admission.*

observed in our cohort of trauma patients. Although an observational study such as the current does not allow to draw conclusions concerning cause and effect, our data suggests that DAMPs play a role in the suppressed state of the immune system. There are some data in support of this. HSP70 is known to induce LPS tolerance in monocytes, an *in vitro* phenomenon showing similarities to *in vivo* immune suppression [21]. Accordingly, we found a inverse relation between plasma HSP70 levels and HLA-DRA expression. Moreover, previous studies have suggested an immunomodulatory role for mtDNA in trauma patients [18, 32]. Of interest, while levels of mtDNA were increased after trauma, this increase was relatively modest. Levels of circulating nDNA were much higher, and unlike mtDNA, correlated with HLA-DRA expression, indicating that mtDNA release is not one of the major factors behind immune suppression in trauma patients. Previous studies that demonstrated much higher mtDNA concentrations in plasma had much smaller patient numbers (n=15 [18] and n=38 [32]) and used only a single 1600g centrifugation step [18, 32]. Chiu et al demonstrated that double centrifugation of plasma (at 1600g and 16000g, as performed in our study) is necessary to remove residual cells, each containing thousands of copies of the mitochondrial genome, making the results from one-spin protocol studies less reliable [33]. One could argue that a difference in injury severity could explain the difference, as Zhang et al included only patients with ISS>25 [18]. However, Lam et al included a majority of patients with ISS<16 [32], making it unlikely that the lower injury severity in our study population (median ISS of 26) explains the lower levels of mtDNA found.

Our study further shows that the early immune response following trauma has a distinct anti-inflammatory phenotype. Plasma levels of the archetypal pro-inflammatory mediator TNF-α were not increased whatsoever and increases in other pro-inflammatory cytokines such as IL-8 and IL-6 were relatively modest and peaked at later time-points. In sharp contrast, the anti-inflammatory cytokine IL-10 was produced rapidly following trauma and already reached peak levels at arrival in the ER. Of interest, in a previous study, trauma patients that were considered "immunoparalytic" based on HLA-DR expression on alveolar macrophages displayed higher IL-10 levels in BAL fluid [34]. IL-10 attenuates the immune response in several ways, for example through inhibition of the production of proinflammatory cytokines, such as TNF-α and IL-6 [35]. In our study, the initial IL-10 peak was followed by a peak in IL-6, which reached highest levels at day 1 following trauma. IL-6 is most renowned for its pro-inflammatory properties, although in trauma it is suggested that continuous IL-6 release accounts for the up-regulation of anti-inflammatory mediators, such as prostaglandin E2, IL-1

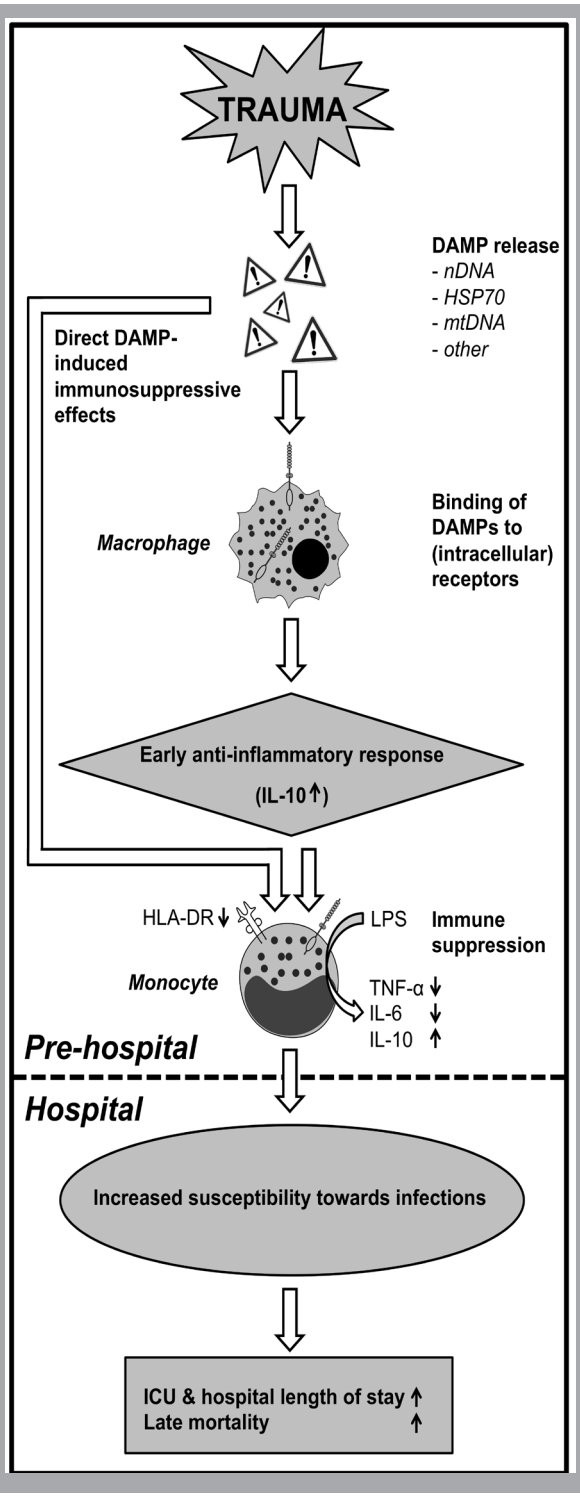



***Figure 4 – Conceptual figure of how DAMP release may lead to increased susceptibility towards infections following trauma*** *Trauma results in the release of DAMPs, including, but not limited to nDNA and HSP70. Subsequently, DAMPs bind to (intracellular) receptors on immune cells such as macrophages, which induces a predominantly anti-inflammatory response characterized by IL-10 release. In turn, this leads to immune suppression, indicated by decreased monocytic HLA-DR expression as well as reduced production of TNF-α/IL-6 and increased production of IL-10 upon ex vivo stimulation with LPS. Alternatively, DAMPs can exert direct immunosuppressive effects, such as HSP70-induced LPS-tolerance in monocytes. All these events take place in the very early (pre-hospital) phase following trauma. In the hospital, aggravated immune suppression is associated with increased susceptibility towards infections, consequent prolonged ICU and hospital length-of-stay, and increased late mortality.*

receptor antagonist, IL-10 and transforming growth factor (TGF)-β and thereby also exhibits anti-inflammatory properties [36, 37]. These findings indicate that immune suppression sets in directly after the injury. The mechanisms initiating this immediate anti-inflammatory response remain to be elucidated, although these findings are in agreement with the current paradigm of the immune response during sepsis. In sepsis, it is now generally accepted that, instead of a previously assumed biphasic inflammatory response, consisting of an initial pro-inflammatory response and a subsequent compensatory anti-inflammatory response, a simultaneously occurring pro- and anti-inflammatory response is present [38]. Others have shown that the production of pro-inflammatory cytokines by leukocytes *ex vivo* stimulated with LPS is severely attenuated following trauma [11, 13]. Herein, we confirm these findings and demonstrate that the production of IL-10 is increased in these patients, with both phenomena already apparent at the trauma scene. This distinct anti-inflammatory phenotype *ex vivo* in the early phase following trauma corroborates our *in vivo* findings.

In keeping with previous work, our data reveal that HLA-DRA expression is decreased following trauma [6, 8, 10, 12, 13]. We importantly extend these findings by showing that this event takes place already before hospital admission and that DAMPs are associated with this phenomenon. The increased IL-10 levels early on following trauma might play a role in the decreased HLA-DRA expression, as IL-10 is known to reduce macrophage function. Furthermore, in accordance with an earlier study [13], we demonstrate that low HLA-DRA levels were associated with decreased production of pro-inflammatory cytokines in response to *ex vivo* stimulation of leukocytes with LPS. Several studies on small cohorts of trauma patients have investigated the relationship between HLA-DR expression and infectious complications [6, 8, 12, 13]. Some have found (trends towards) associations between low HLA-DR expression and infections [6, 13], while others have not [12]. One study showed that reduced expression of HLA-DR on alveolar macrophages, but not on circulating leukocytes was associated with nosocomial pneumonia [8]. However, concerning the relation with outcome and/or development of infections, studies in trauma patients, septic patients, and in a cohort of ICU patients with various conditions have revealed that recovery of HLA-DR, rather than absolute values, is important [10, 29, 31]. In keeping with this, we found that a further decrease of HLA-DRA expression between admission and day 3 predicts development of infections. Taken together, these data suggest that aggravated immune suppression following the initial hit increases the risk of infection after trauma. Nevertheless, the anti-inflammatory phenotype present directly after

trauma might also have beneficial effects through limiting excessive inflammation and thereby organ damage. As such, whether this phenotype is solely detrimental or has homeostatic features as well remains to be determined.

Our study has several limitations. First, inherent to this type of study, a substantial number of patients were lost to follow-up, e.g. because of discharge from the hospital or transfer to another hospital (in most cases due to recovery), or death (although mortality was low in our cohort). Therefore, if alterations in parameters observed initially in patients amend in those who recover, this could be missed. However, this does not affect the main conclusions of the manuscript as these are based on data obtained at early time-points or and data of a subgroup of patients with a follow up of several days. Another weakness of the current work typical for the multi-trauma patient population studied is the heterogeneity of the patients. Second, the use of plasma nDNA levels as a marker of general DAMP release could be debated, as it is possible that specific DAMPs display other release or clearance patterns and not necessarily follow plasma nDNA concentrations. Future studies focusing on the extensive range of DAMPs important in trauma could shed more light on this phenomenon and the importance of individual DAMPs in trauma.
Third, we used expression of the HLA-DRA gene in whole blood leukocytes as a marker of immune suppression, while most studies have used HLA-DR expression on the surface of monocytes determined using flow cytometry for this purpose. Flow cytometric analysis requires rapid analysis after sampling and the constant availability of a flow cytometer, which was not feasible in our setting, especially with regard to the samples obtained at the trauma scene. Nevertheless, the use of gene expression data is a limitation, as posttranscriptional effects can also affect HLA-DRA expression. Furthermore, next to monocytes, other cells present in whole blood may also express the HLA-DRA gene to various extents and/or may exhibit different kinetics of expression, although there is little known on this subject in the context of immune suppression. In several studies in septic patients, a population which is, similar to ours, highly heterogeneous and likely exhibiting profound changes in leukocyte counts and differentiation over time, HLA-DRA gene expression was not corrected for leukocyte counts/differentiation [27-29, 39]. Also, we do not have adequate data on daily leukocyte counts and differentiation in our cohort, as these were not regularly measured in the majority of patients. Nevertheless, the aforementioned studies in septic patients have shown that gene expression of HLA-DRA correlates well with flow cytometric analysis of mHLA-DR, with correlation coefficients ranging from 0.74 to 0.84 [27-29], although in one of these, a more moderate, but still highly significant correlation coefficient of 0.53

was found [39]. Therefore, we feel qPCR analysis of HLA-DRA in our study is a reliable indicator of HLA-DR expression and immune suppression. Yet, we acknowledge that the lack of data on leukocyte counts is a limitation, because, next to possible effects on HLA-DR gene expression data, lymphopenia also represents a hallmark of immune suppression.

Finally, our control group consisted of solely young male volunteers. Especially with regard to the phenomenon of immunosenescence [40], this could have biased our results. However, when we compared levels of DAMPs and immunological parameters across 5 age categories (<28 [n=33], 28-42 [n=33], 43-56 [n=34], 57-70 [n=32], and >70 [n=32]), or between males and females within our patient cohort, we found no differences in any of the parameters at any of the measured time-points. Also, we did not assess the functionality of the adaptive immune system, for instance using functional assays such as proliferation or cytokine release by T-cells.

In conclusion, we demonstrate that trauma results in release of DAMPs, and that this is associated with to an acute predominantly anti-inflammatory response and a suppressed state of the immune system. In trauma patients, these events take place already before hospital admission and the observed immune suppressed state is not preceded or accompanied by a pronounced pro-inflammatory phase. Aggravated immune suppression, as indicated by further decrease of HLA-DRA expression is associated with the development of nosocomial infections in this patient population.

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# Chapter 3

## The involvement of danger-associated molecular patterns in the development of immunoparalysis in cardiac arrest patients

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## Abstract

**Objective** After cardiac arrest, patients are highly vulnerable towards infections, possibly due to a suppressed state of the immune system called immunoparalysis. We investigated if immunoparalysis develops following cardiac arrest and whether the release of danger associated molecular patterns (DAMPs) could be involved.

**Design** Observational study.

**Setting** ICU of a university medical center.

**Patients** Fourteen post-cardiac arrest patients, treated with mild therapeutic hypothermia for 24 hrs and 11 control subjects.

**Measurements and main results** Plasma cytokines showed highest levels within 24 hours after cardiac arrest and decreased during the next 2 days. In contrast, *ex vivo* production of cytokines IL-6, TNF-α, and IL-10 by LPS-stimulated leukocytes was severely impaired compared with control subjects, with most profound effects observed at day 0, and only partially recovering afterwards. Compared with incubation at 37°C, incubation at 32°C resulted in higher IL-6 and lower IL-10 production by LPS-stimulated leukocytes of control subjects, but not of patients. Plasma nuclear DNA, used as a marker for general DAMP release, and the specific DAMPs EN-RAGE and HSP-70 were substantially higher in patients at days 0 and 1 compared with control subjects. Furthermore, plasma HSP70 levels were negatively correlated with *ex vivo* production of inflammatory mediators IL-6, TNF-α, and IL-10. EN-RAGE levels only showed a significant negative correlation with *ex vivo* production of IL-6 and TNF-α, and a borderline significant inverse correlation with IL-10. No significant correlations were observed between plasma nDNA levels and *ex vivo* cytokine production.

**Conclusions** Release of DAMPS during the first days after cardiac arrest is associated with the development of immunoparalysis. This could explain the increased susceptibility towards infections in cardiac arrest patients.

## Introduction

Survival after cardiac arrest is low, even after return of spontaneous circulation is achieved [1-4]. This is partly due to the development of the post-cardiac arrest syndrome, a condition demonstrating similarities to severe sepsis and characterized by a systemic ischemia/reperfusion response, brain injury, myocardial dysfunction, and persistent precipitating disease [5, 6]. Furthermore, post-cardiac arrest patients are highly vulnerable towards infections, which occur in 46 to 76% of patients [7-9]. Leukocytes of patients after cardiac arrest show a diminished capacity to produce pro-inflammatory cytokines in response to *ex vivo* stimulation with bacterial antigens, and a reduced expression of monocytic HLA-DR, observations consistent with the development of immunosuppression [10-12]. This immunosuppressive phenotype in cardiac arrest patients bares similarities to sepsis-induced immunoparalysis, a profoundly suppressed state of the immune system following the initial infection which accounts for the increased risk for secondary infections in septic patients [11-13]. Likewise, the increased vulnerability towards infections after cardiac arrest might be due to the development of immunoparalysis. However, hitherto only one small study to date has investigated this phenonemon [10] and it is unknown which factors contribute to the development of immunoparalysis in these patients.

Danger associated molecular patterns (DAMPs) can be actively released by ischemic cells as danger signals or originate from damaged or dead cells as debris [14, 15]. A large number of different DAMPs have been identified, of which several have been shown to exert immunomodulatory effects. For instance, mitochondrial DNA (mtDNA) can trigger immune responses via Toll-like receptor 9 [16, 17], EN-RAGE, a DAMP from the S100 protein family, modulates inflammation via inhibition of matrix metalloproteinases and chemotaxis [18], and HSP (Heat Shock Protein) 70 exerts anti-inflammatory properties [19, 20]. Also, previous studies have indicated that free nuclear DNA (nDNA) in plasma is a marker for cell damage or death, as it is one of the many cell components released if a cell is ruptured [21, 22]. Therefore, it might be an indicator of DAMP release in general. The role of DAMPs in modulation of the immune response post-cardiac arrest has not been investigated.

Another factor that might play a role in development of post-cardiac arrest immunoparalysis is hypothermia, which has been identified as an independent risk factor for the development of infections [8]. However, no effects of mild

therapeutic hypothermia (MTH) on *ex vivo* leukocyte responses to bacterial antigens and HLA-DR expression were found [10]. Interestingly, hypothermia has been shown to induce HSP70 [23-25].

The aim of this study was to assess if DAMP release in general, reflected by plasma levels of nDNA, and levels of the specific DAMPs (mtDNA, EN-RAGE, and HSP70) are increased following cardiac arrest. Furthermore, we assessed whether DAMPs are associated with the development of immunoparalysis, reflected by *ex vivo* leukocyte responses. Finally, we investigated if possible immunosuppressive effects of hypothermia in leukocytes obtained from cardiac arrest patients can be reversed by *ex vivo* rewarming.

## Materials and Methods

### Study population

We performed a prospective observational study in 14 patients successfully resuscitated from an out-of-hospital cardiac arrest and treated with MTH for 24 hours. The study has been carried out in the Netherlands in accordance with the applicable rules concerning the review of research ethics committees and informed consent. The local ethics committee reviewed the study and waived the need for approval. All patients or legal representatives were informed about the study details. All study procedures were conducted in accordance with the declaration of Helsinki including current revisions and Good Clinical Practice guidelines.

All patients of 18 years or older were eligible for the study if they were comatose (Glasgow Coma Scale ≤ 6) after return of spontaneous circulation and if the suspected cause of the arrest was of cardiac origin. Patients were excluded in case of thrombolytic therapy, refractory cardiogenic shock, an anticipated life expectancy < 24 hrs, an active infection at the time of inclusion, hematological disease, use of immunosuppressive drugs, or suffering from other conditions known to influence the immune response (e.g. auto-immune diseases).

Control samples were obtained from volunteers (n=11, 6 male/5 female, median age 65 [range 36-76]) visiting the out-patient clinic for anesthesiology intake and screening prior to elective surgery or elective spectroscopy. Written informed consent was obtained from all of these volunteers before blood sampling.

### Patient management

All patients were admitted to the ICU of a tertiary care university hospital in Nijmegen, The Netherlands, and treated according to the local protocol. If

necessary, a coronary angiogram and a percutaneous coronary intervention were performed before admission to the ICU. All patients were cooled to 32-34°C by rapid infusion of 30 ml/kg bodyweight of cold Ringer's lactate at 4°C followed by external cooling using two water-circulating blankets (Blanketroll II, Cincinatti Subzero, The Surgical Company, Amersfoort, The Netherlands). Temperature was measured continuously with a rectal temperature probe (YSI Incorporated 401, vd Putte Medical, Nieuwegein, The Netherlands) and maintained at 32-34°C for 24 hrs, followed by passive rewarming to normothermia (defined as 37°C). All patients were sedated with midazolam and/or propofol and morphine during hypothermia. Sedation and analgesics were stopped as soon as the body temperature was ≥ 36.5°C. In case of shivering, patients were paralyzed using intravenous bolus injections of rocuronium.

All patients were intubated and mechanically ventilated aiming at a $PaO_2$ > 75 mm Hg and a $PaCO_2$ between 34 and 41 mm Hg. The radial or femoral artery was cannulated for monitoring of blood pressure and sampling of arterial blood. Mean arterial blood pressure was maintained between 80-100 mmHg, and diuresis was aimed at > 0.5 ml/kg/hr. If necessary, patients were treated with volume infusion and dobutamine and/or (nor)epinephrine intravenously.

Serum concentrations of sodium, potassium and magnesium were maintained within the normal range. All patients were treated with continuous insulin infusion therapy to keep blood glucose levels between 6-8 mmol/l. Haemoglobin concentration was kept ≥ 6.0 mmol/l. None of the patients were treated with anti-inflammatory drugs or steroids.

**Sample and data collection**

Demographic data and clinical parameters were collected from electronic patient files. Blood was sampled from the arterial catheter within 24 hours after admission (day 0, during MTH), and at day 1 and 2. Lithium-heparin (LH) anticoagulated blood was obtained for *ex vivo* stimulation experiments, which were performed immediately after withdrawal. Ethylenediaminetetraacetic acid (EDTA) anticoagulated blood was immediately centrifuged after withdrawal at 1,600 x g at $4^0$C for 10 minutes, after which plasma was stored at -$80^0$C until further analysis. Plasma for real time quantitative PCR (qPCR) analysis was centrifuged again at 16,000 x g at $4^0$C for 10 minutes to remove potential remaining cells and debris. The supernatant was stored at -$80^0$C until further analysis.

**Nuclear and mitochondrial DNA quantification in plasma**

Plasma from double-centrifuged EDTA anticoagulated blood was diluted 1:1

with phosphate buffered saline solution (PBS) after which DNA was isolated using the QIAamp DNA Blood Midi Kit (Qiagen, Valencia, CA, USA), using the 'Spin Protocol' as described by the manufacturer. Isolated DNA was stored at -20$^0$C until further analysis. qPCR was performed using the CFX Real-Time PCR Detection system (Bio-Rad Laboratories, Hercules, CA, USA). The GAPDH gene was used for amplification of nDNA (Forward 5'-AGCACCCCTGGCCAAGGTCA-3', Reverse 5''-CGGCAGGGAGGAGCCAGTCT-3'). For mtDNA quantification, the following primers were used: forward 5'-GCCCCAACGTTGTAGGCCCC-3' and reverse 5'AGCTAAGGTCGGGGCGGTGA-3'. The PCR reaction mixture consisted of 5 µl isolated DNA, 5.5 µl nuclease free water, 12.5 µl iQ SYBR Green PCR Master Mix (Bio-Rad Laboratories, Hercules, CA, USA) and 1 µl forward and reverse primer. Samples were analyzed in duplicate and a fresh aliquot of DNA isolated from blood obtained from a healthy volunteer was used in each plate as a calibrator. Plasma nDNA and mtDNA quantities are expressed as fold change relative to the expression of the same gene in the calibrator sample.

**Plasma cytokine and DAMP concentrations**

Plasma concentrations of Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-2, IL-4, IL-6, IL-8, IL-10, IL12p70, IL-1β, Interferon (IFN)-γ, IL-1 receptor antagonist (IL-1RA), and Monocyte Chemoattractant Protein (MCP)-1 were analyzed batchwise using a simultaneous Luminex assay according to the manufacturer's instructions (Milliplex; Millipore, Billerica, MA, USA). Plasma concentrations of HSP70/HSPA1A and EN-RAGE/S100A12 were determined batchwise using ELISA according to the manufacturer's instructions (R&D systems, Minneapolis, MN, USA and MBL international, Woburn, MA, USA, respectively).

***Ex vivo* cytokine production**

Leukocyte cytokine production capacity was determined by challenging whole blood from the patients with LPS *ex vivo* using an in-house developed system with pre-filled tubes described in detail elsewhere [26]. Briefly, 0.5 mL of blood was added to tubes pre-filled with 2 mL RPMI culture medium or 2 mL culture medium supplemented with 12.5 ng/mL LPS (end concentration of LPS: 10 ng/mL). Tubes were incubated at 37°C (patient samples at days 0, 1, and 2, and samples from controls) and 32°C (patient samples at day 0 and samples from controls) for 24 hours, centrifuged, and supernatant was stored at -80°C until analysis. Concentrations of TNF-α, IL-6 and IL-10 were determined by ELISA, according to the manufacturer's instructions (R&D systems, Minneapolis, MN, USA).

Automated leukocyte counts differentiation were performed in EDTA

anticoagulated blood using routine analysis methods (flow cytometric analysis on a Sysmex XE-5000). *Ex vivo* produced cytokines were normalized for monocyte count since these are the main producers of cytokines in whole blood stimulations [27].

### Statistical analysis

Data are expressed as mean±SEM or median [range], according to their distribution as determined using the Shapiro-Wilk test. Kruskal Wallis with post hoc Dunn's test (plasma cytokines or DAMP levels) or one-way ANOVA with post-hoc Dunn's test (*ex vivo* cytokine production) were used to test for significant differences between controls and patients at days 0, 1 and 2. According to the distribution of the data, Mann-Whitney U or Student's T-tests were performed for every outcome variable in both patients (data from day 0) and controls to investigate gender-based differences. Correlation coefficients were calculated using Bland and Altman's method for calculating correlation coefficients with repeated observations [28]. Statistical analysis was performed using Graphpad Prism 5 software (Graphpad Software, La Jolla, USA) and SPSS Statistics 20 (IBM, NY, USA). A p-value of $<0.05$ was considered statistically significant.



## Results

### Patient characteristics

Patients characteristics are summarized in Table 1. Norepinephrine and midazolam was used in all patients. Furthermore, sufentanil was used in 13/14 patients, dobutamin in 7/14, milrinon in 3/14, and metoprolol in 11/14.

No difference between controls and patients were observed in plasma cytokine concentrations of IFN-γ, IL-2, IL-4, IL-12p70, and IL-1β. Plasma IL-10, IL-1RA, IL-6, IL-8, MCP-1, and TNF-α showed highest plasma levels at day 0, and levels were significantly higher compared with controls on days 0 and 1 (Figure 1). In the next two days, plasma levels gradually decreased and returned to concentrations comparable with those found in controls on day 2.

No gender-based differences were observed for any of the outcome variables in both patients (data from day 0) and controls.

### Plasma DAMP levels

Compared with controls, plasma levels of HSP70, EN-RAGE and nDNA were significantly increased on day 0 and 1. Plasma mtDNA levels were only slightly, but significantly increased at day 0 and 2 in cardiac arrest patients compared with controls (Figure 2).

| | |
|---|---|
| Gender | Male n=10 (71.4%)<br>Female n=4 (28.6%) |
| Age (years, median[range]) | 63.5 [38-84] |
| BMI*(kg/m$^2$, median[range]) | 24.8 [23.0-28.9] |
| Time to ROSC (min, median[range]) | Unknown: n=5<br>20 [10-40] |
| SAPS (median[range]) | 66.5 [26-103] |
| APACHE (median[range]) | 24 [15-39] |
| Initial rhythm | VF n=12 (85.7%)<br>asystoly n=2 (14.3%) |
| In-hospital mortality | N=5 (35.7%) |
| Body temperature (°C, median [range]) | Day 0: 33.7 [32.0-35.1]<br>Day 1: 37.1 [32.5-38.3]<br>Day 2: 37.4 [37.0-38.0] |

***Table 1 - Patient characteristics*** ** BMI was unknown in 2 patients*

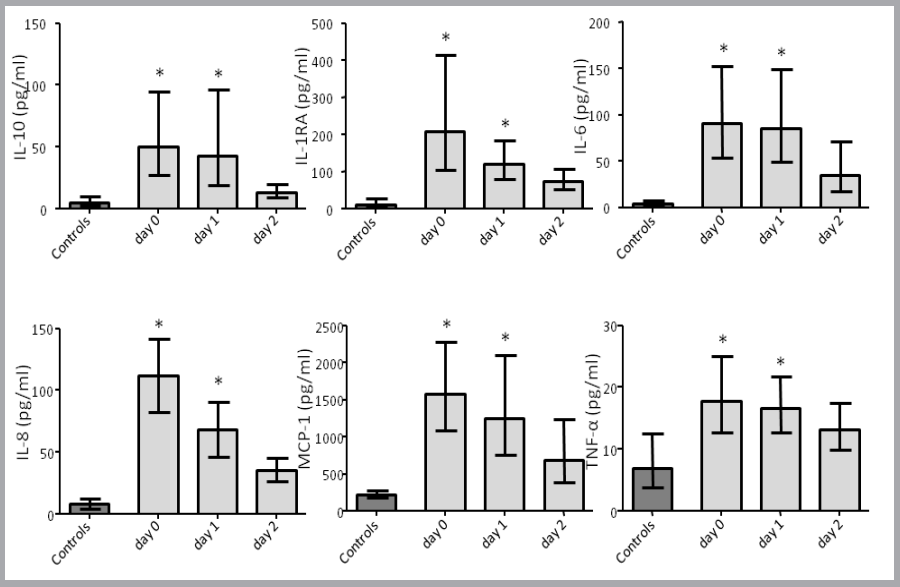


***Figure 1 – Plasma cytokines*** *Plasma cytokine concentrations in controls and cardiac arrest patients on days 0, 1, and 2. Data are depicted as geometric mean±95% CI. * indicates p<0.05 compared with controls calculated using Kruskal-Wallis with post-hoc Dunn's test.*

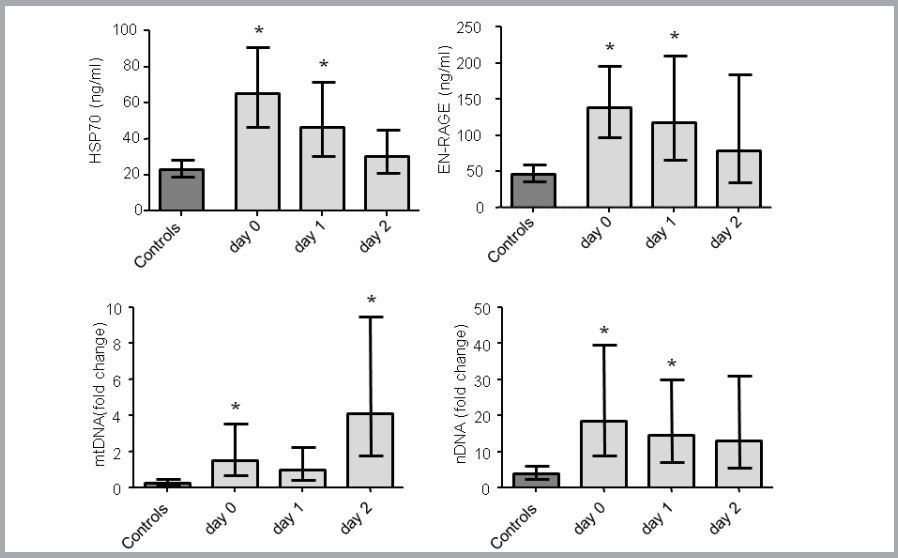


***Figure 2 – DAMP levels*** *Plasma levels of HSP70 and EN-RAGE, nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) in controls and cardiac arrest patients on days 0, 1, and 2. Data are depicted as geometric mean±95% CI. * indicates p<0.05 compared with controls calculated using Kruskal-Wallis with post-hoc Dunn's test.*

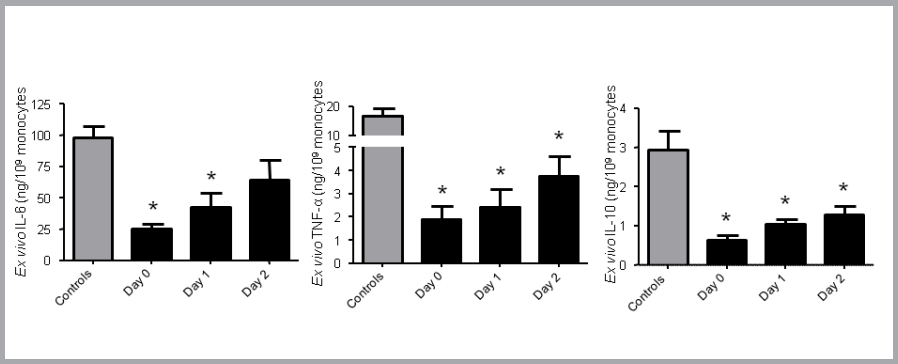


***Figure 3 – Ex vivo cytokine production*** *IL-6, TNF-α, and IL-10 production by leukocytes stimulated with LPS ex vivo in controls and cardiac arrest patients on days 0, 1, and 2. Data are expressed as mean±SEM ng cytokine/$10^9$ monocytes. * indicates p<0.05 compared with controls calculated using one-way ANOVA with Bonferroni post-hoc test.*

### *Ex vivo* cytokine production

In cardiac arrest patients, the *ex vivo* production of cytokines IL-6, TNF-α, and IL-10 was severely impaired in comparison with controls (Figure 3). This effect was most pronounced for the pro-inflammatory cytokine TNF-α. The suppressed response was most profound at day 0 and only partially recovered in the following days.

In controls, incubation at 37°C resulted in reduced IL-6 production (p=0.0002) and a trend towards reduced TNF-α production (p=0.14), while IL-10 production was increased (p=0.02) compared with incubation at 32°C. No differences were observed between both incubation temperatures in the patient group (Figure 4). *Ex vivo* TNF-α production at 37°C at day 0 was significantly decreased in patients treated with dobutamin compared with patients not receiving dobutamin (1.03±0.19 vs 3.35±0.12 ng/$10^9$ monocytes, p=0.004).

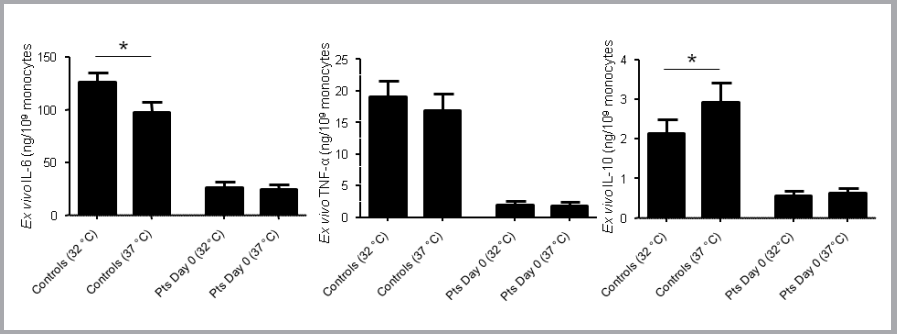


***FIgure 4 – Ex vivo incubation 32°C or 37°C*** *IL-6, TNF-α, and IL-10 production by leukocytes stimulated with LPS ex vivo at 32°C or 37°C in controls and cardiac arrest patients at day 0 (Pts Day 0). Data are expressed as mean±SEM ng cytokine/$10^9$ monocytes. * indicates p<0.05 according to paired T-tests.*

### Correlations between DAMPs and *ex vivo* cytokine production

Plasma HSP70 levels were negatively correlated with ex vivo production of inflammatory mediators IL-6, TNF-α and IL-10 (Figure 5, panels A, B and C). EN-RAGE levels showed a significant negative correlation with *ex vivo* production of IL-6 and TNF-α, and a borderline significant inverse correlation with IL-10 (Figure 5, panels D, E, and F). No significant correlations were observed between plasma nDNA and *ex vivo* cytokine production (r=-0.34, p=0.21; r=-0.35, p=0.20 and r=-0.20, p=0.46, for IL-6, TNF-α and IL-10, respectively).

Plasma levels of nDNA significantly correlated with plasma HSP70 levels (r=0.50, p=0.03), but not with EN-RAGE or mtDNA levels (r=0.37, p=0.12 and r=-0.23, p=0.41, respectively).

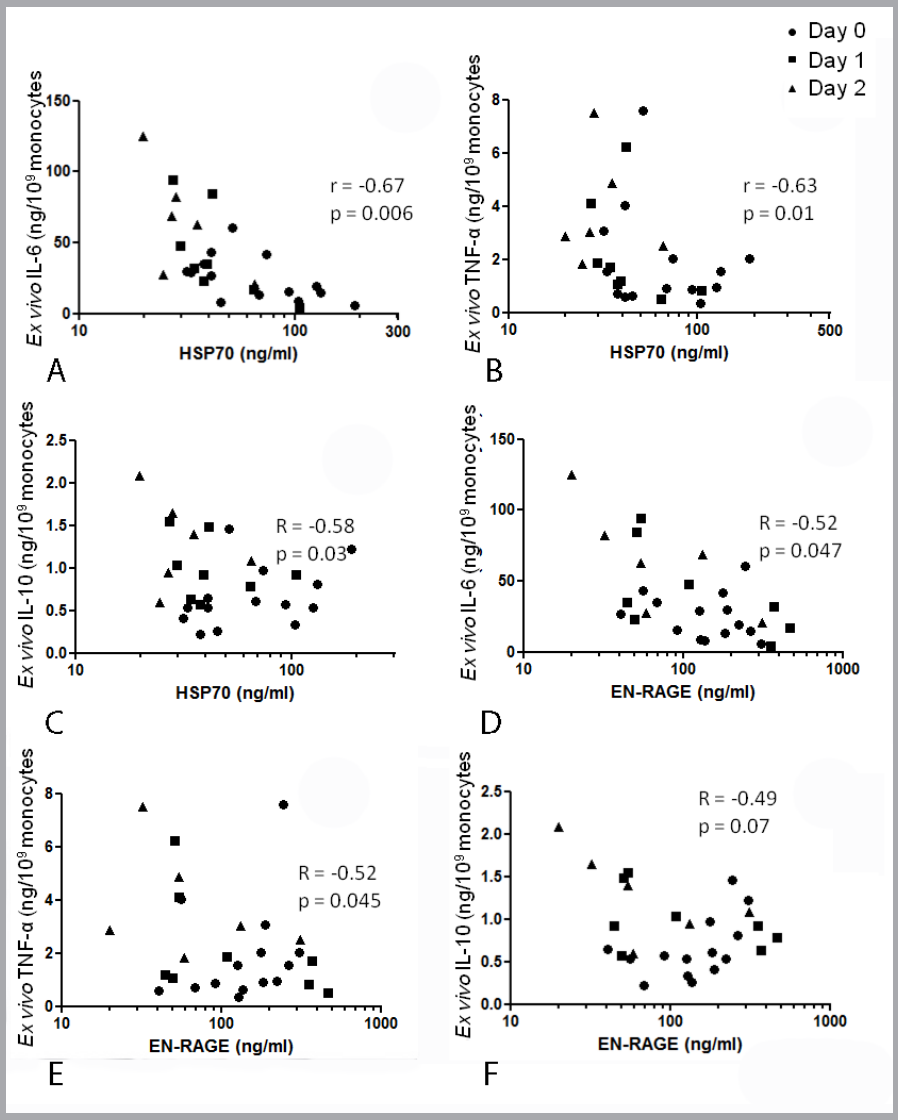


***Figure 5 – Correlations between circulating DAMPs and ex vivo cytokine production***

*R and p values calculated using spearman correlation on combined data from days 0, 1 and 2.*

## Discussion

In the present study, we demonstrate that plasma levels of nDNA, a marker for general DAMP release, and the specific DAMPs HSP70 and EN-RAGE are increased within 24 hours after cardiac arrest and remained elevated during the following days. Furthermore, we confirm increased plasma cytokine levels within 24 hours after cardiac arrest and a subsequent decline in the following days. In contrast, production of cytokines by leukocytes stimulated *ex vivo* displayed an inverse pattern: a severely impaired response at day 0 followed by partial recovery. These findings are indicative of immunoparalysis in cardiac arrest patients. Moreover, circulating levels of EN-RAGE and especially HSP70 inversely correlated with *ex vivo* cytokines production.

Our study suggests that during the first days of hospital admission after cardiac arrest, ongoing cell damage results in the continuous release of DAMPs in the circulation and that these DAMPS account for the observed immunoparalysis. Immediately after cardiac arrest, the increased plasma DAMP levels probably result from direct cardiac cell damage following ischemia/reperfusion injury. However, the uptake and clearance of dead cells is a highly efficient process mediated by macrophages that results in complete clearance of plasma DNA within 24 hours [29]. Because plasma nDNA, HSP70, and EN-RAGE remained elevated during the first two days after cardiac arrest, ongoing cell damage during hospital admission is likely to induce continuous DAMP release in these patients. Alternatively, extensive cell death has been suggested to induce apoptosis of macrophages after phagocytosis of the dead cells or cellular debris, which could lead to the release of nuclear contents (both of the phagocytosed cells and the macrophage itself) into the circulation [29]. Although mtDNA gained much attention as a DAMP to induce inflammation in e.g. trauma patients [17], our study demonstrates that mtDNA, in contrast to HSP70 and EN-RAGE, is not one of the DAMPs released in large quantities in post-cardiac arrest patients.
The observed difference between plasma nDNA and mtDNA concentrations in this study is remarkable, as one would expect that cells release both substances when damaged. Three possible mechanisms could explain these differences. First, mitochondria could be cleared before cell death occurs via a process called mitophagy [30]. Second, intact mitochondria instead of free mtDNA could be released into the plasma. A third and final possibility is that mtDNA could be cleared at a much higher rate compared with nDNA, because of rapid recognition and phagocytosis due to its bacterial-like appearance. Additional studies are

warranted to gain more insight in the mechanisms of cell death post-cardiac arrest and the release of mitochondria or mtDNA in this process.

Our data demonstrate that circulating leukocytes display a suppressed phenotype during the first days after cardiac arrest, reflected by decreased production of cytokines upon stimulation with LPS. *Ex vivo* cytokine production was inversely related to plasma levels of EN-RAGE and especially HSP70, implicating that these DAMPs contribute to the observed compromised immune function. HSP70 showed the strongest inverse relationship with pro-inflammatory cytokines production and interestingly, this HSP has been shown to induce endotoxin tolerance in monocytes *in vitro* [20]. Endotoxin tolerance is characterized as a reduced capacity to respond to LPS stimulation following a previous exposure to LPS [20, 31, 32], and has many similarities to immunoparalysis [33]. The fact that, despite the apparent immunosuppression, plasma cytokines are still elevated compared with controls and the lack of a relationship between nDNA levels and the *ex vivo* immune response, suggests that both immunostimulatory and immunosuppressive DAMPs are released. Taken together, these results indicate cardiac arrest patients show signs of immunoparalysis, possibly as a result of DAMP release, of which especially HSP70 appears to play an important role. The immunoparalysis observed has been previously described in sepsis patients [34, 35]. In this respect, the declining plasma cytokine levels in spite of still-elevated DAMP levels on days 1 and 2 might reflect immunoparalysis of the tissue-resident compartment, which has been shown before to remain immunoparalytic for much longer than circulating leukocytes [36]. No relation with DAMP release has previously been described in this patient group, although multiple studies in trauma patients, with major DAMP release at the time of injury, indicates a relationship between the release of DAMPs and immunoparalysis [37-39].

Worldwide, MTH is frequently employed in patients after cardiac arrest and is part of the standard care in our hospital. Interestingly, MTH has been suggested to attenuate pro-inflammatory cytokine production [40, 41], and could therefore contribute to the development of immunoparalysis in these patients. This is however not supported by a recent study that demonstrated no differences in *ex vivo* cytokine responses or HLA-DR expression between patients treated with and without MTH [10]. Studies into the effects of temperature on *ex vivo* LPS-induced cytokine production showed conflicting results as well [42, 43]. We did not find any effects of *ex vivo* rewarming of leukocytes obtained from patients during MTH on cytokine production. However, we did observe an effect of incubation temperature

on *ex vivo* cytokine production in the control group. These findings might suggest that the phenotype of leukocytes is altered by *in vivo* hypothermia, and that this effect cannot be reversed by *ex vivo* rewarming of cells. This is nevertheless not in line with the findings of enhanced pro-inflammatory cytokine production upon incubation at 32°C, a phenotype dissimilar to that observed in cardiac arrest patients. Therefore, MTH appears not to be the main cause of reduced immune function in these patients. Interestingly, HSP70, which correlates with the extent of immunoparalysis in the present study, is induced by hypothermia [25]. Along these lines, the profoundly increased levels of HSP70 on day 0 might be partly explained by the MTH-treatment at this time-point.

This study has several limitations. The medication used by our patients might influence the immunoparalysis observed. Patients receiving dobutamin displayed lower *ex vivo* TNF-α production compared to patients not treated with dobutamin. However, the fact that the untreated group still demonstrated a vast decrease in *ex vivo* TNF-α production compared with controls suggest that the role of dobutamin in the development of immunoparalysis is limited. Moreover, the study population is highly heterogeneous and the sample size does not allow a multiple regression analysis including multiple DAMPs or patient factors, such as age. Due to the small sample size, no relevant clinical outcome measures, such as infections or survival, could be assessed as well. Finally, as this is a correlative observational study, no definite conclusions can be drawn concerning possible mechanisms behind DAMP release, e.g. ischemia/reperfusion or hypoxia, and the related immunoparalysis.

## Conclusions

In conclusion, DAMPs are continuously released during the first days after cardiac arrest, and levels of the specific DAMPs EN-RAGE and especially HSP70 are inversely correlated with the extent of immunoparalysis. This suggests that DAMPs in general, and particularly HSP70, are involved in development of immunoparalysis. The observed immunoparalysis could play an important role in the susceptibility towards infections in cardiac arrest patients but could also represent a protective mechanism to limit secondary tissue damage.

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# Chapter 4

## DAMP release following chemotherapy does not induce immunoparalysis in leukemia patients

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*Submitted*

## Abstract

Chemotherapy may result in the release of Danger Associated Molecular Patterns (DAMPs), which can cause deactivation of the immune system, a phenomenon known as immunoparalysis. We investigated if DAMPs are released following chemotherapy and their relationship with markers of immunoparalysis in leukemia patients. In 6 patients with acute myeloid leukemia or myelodysplastic syndrome, several DAMPs and inflammatory cytokines in the circulation as well as markers of immunoparalysis (leukocyte cytokine production capacity and HLA-DR expression) were determined before and 4-6 hours, 2, 4, 6, and 8 days following chemotherapy initiation. Immunoparalysis markers were also determined in 12 healthy control subjects. Compared with healthy controls, plasma levels of nuclear DNA (a marker of general DAMP release) in patients were profoundly increased before chemotherapy and further increased 4-6 hours afterwards, while the specific DAMP mitochondrial DNA only showed a trend towards increased levels. Circulating cytokine levels did not change following chemotherapy. Finally, leukocyte cytokine production capacity and HLA-DR expression were similar between patients and healthy controls until day 4, when leukocytes were virtually absent in the circulation. In conclusion, in the early phase following chemotherapy in leukemia patients, increased DAMP release does not induce immunoparalysis.

## Introduction

The standard treatment for acute haematological malignancies usually includes chemotherapy [1]. This treatment is aimed to reduce tumour burden and to induce long-term remission, but at the same time renders patients increased vulnerable for infections, both from invading pathogens and from endogenous flora or viral reactivations [2]. It is well known that chemotherapy-induced immune cell destruction is the major contributor to this decreased immune function [3]. However, defective immune function in remaining cells has also been described, for example in neutrophils and T-lympohocytes [4, 5].



Chemotherapy used to treat haematological malignancies may result in the release of so-called Danger Associated Molecular Patterns (DAMPs) [6]. These DAMPs elicit an immune response in a similar manner as Pathogen Associated Molecular Patterns (PAMPs) derived from invading pathogens in infectious diseases such as sepsis [7, 8], but may subsequently also result in deactivation of the immune system, a phenomenon known as immunoparalysis in the sepsis field [9, 10]. Two major hallmarks of immunoparalysis that are related to immune cell dysfunction are attenuated production of cytokines by leukocytes *ex vivo* stimulated with PAMPs such as LPS, and decreased leukocyte HLA-DR expression [9, 11]. As such, DAMP-induced leukocyte immune dysfunction could contribute to the decreased function of the remaining immune cells in leukemia patients treated with chemotherapy.

The aims of this study were to quantify the amount of DAMPs in the circulation before and after initiation of chemotherapy in patients with acute leukemia, and to assess leukocyte cytokine production capacity and HLA-DR expression in these patients. This enabled us to establish the relationship between DAMP levels and immunoparalysis.

## Materials and Methods

### Study population

We performed a prospective observational study in 6 patients aged >18 years and diagnosed for the first time with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS). Exclusion critera: active infection at the time of inclusion, use of immunosuppressive drugs, suffering from other conditions known to influence the immune response (e.g. auto-immune diseases).

The study was carried out in the Netherlands in accordance with the applicable rules concerning the review of research ethics committees and informed consent.

All study procedures were conducted in accordance with the declaration of Helsinki including current revisions and Good Clinical Practice guidelines.
Control samples (n=12) were obtained from healthy male volunteers (median age 22 [range 19-27]) participating in the control group of an experimental endotoxemia trial [12] at baseline, before administration of endotoxin. Written informed consent was obtained from all volunteers prior to screening and inclusion in the study.

**Sample and data collection**

Demographic data and clinical parameters were collected from electronic patient files. Blood was sampled from the venous catheter at baseline on day 1 (before start of chemotherapy), 4-6 hours after start of chemotherapy, and at days 2, 4, 6 and 8. Lithium-heparin (LH) anticoagulated blood was obtained for *ex vivo* whole blood stimulation experiments, which were performed immediately after withdrawal. Ethylenediaminetetraacetic acid (EDTA) anticoagulated blood was immediately centrifuged after withdrawal at 1,600 x g at $4^0$C for 10 minutes, after which plasma was stored at -$80^0$C until cytokine analysis. Plasma for nDNA and mtDNA quantification by real time quantitative PCR (qPCR) was centrifuged again at 16,000 x g at $4^0$C for 10 minutes to remove potential remaining cells and debris. The supernatant was stored at -$80^0$C until analysis. Blood for HLA-DR quantification by qPCR was sampled in Paxgene blood RNA tubes (Qiagen, Valencia, CA, USA) and stored according to the manufacturer's instructions.

**DAMP quantification in plasma**

A large variety of different (and probably many yet unidentified) DAMPs are released in case of cell damage [7]. Therefore, we measured general markers of cell damage (plasma nuclear DNA [nDNA], plasma lactate dehydrogenase [LDH], and urate) as indicators of general DAMP release [13]. Moreover, we quantified plasma mitochondrial DNA (mtDNA), a known DAMP that can trigger Toll-Like receptor (TLR) 9 [14].
Plasma from double-centrifuged EDTA anticoagulated blood was diluted 1:1 with phosphate buffered saline solution (PBS), after which DNA was isolated using the QIAamp DNA Blood Midi Kit (Qiagen, Valencia, CA, USA), using the 'Spin Protocol' as described by the manufacturer. Isolated DNA was stored at -$20^0$C until further analysis. qPCR was performed using the CFX Real-Time PCR Detection sytem (Bio-Rad Laboratories, Hercules, CA, USA). The GAPDH gene was used for amplification of nDNA (Forward 5'-AGCACCCCTGGCCAAGGTCA-3', Reverse 5"-CGGCAGGGAGGAGCCAGTCT-3'). For mtDNA quantification, the

following primers were used: forward 5'-GCCCCAACGTTGTAGGCCCC-3' and reverse 5'AGCTAAGGTCGGGGCGGTGA-3'. The PCR reaction mixture consisted of 5 µl isolated DNA, 5.5 µl nuclease free water, 12.5 µl iQ SYBR Green PCR Master Mix (Bio-Rad Laboratories, Hercules, CA, USA) and 1 µl forward and reverse primer. Samples were analyzed in duplicate and DNA isolated from blood obtained from the same healthy volunteer was used in each plate as a calibrator. Plasma nDNA and mtDNA quantities are expressed as fold change relative to the calibrator sample.

Plasma LDH and urate concentrations were determined using routine analysis methods also used for patient samples in clinical practice (ARCHITECT chemistry analyzer).



***Ex vivo* whole blood stimulation**

Leukocyte cytokine production capacity was determined by stimulating whole blood with LPS *ex vivo* using an in-house developed system with pre-filled tubes described in detail elsewhere [15]. Briefly, 0.5 mL of blood was added to tubes pre-filled with 2 mL culture medium or 2 mL culture medium supplemented with 12.5 ng/mL LPS (end concentration of LPS: 10 ng/mL). Cultures were incubated at 37° for 24 hours, centrifuged at 2000 x g for 10 minutes at 4 $^{0}$C, and supernatants were stored at -80°C until analysis.

**Cytokine analysis**

Plasma concentrations of Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-6, IL-10, IL-8, Interferon (IFN)-γ, and IL-1 receptor antagonist (IL-1RA) were analyzed batchwise using a simultaneous Luminex assay according to the manufacturer's instructions (Milliplex; Millipore, Billerica, MA, USA). Concentrations of TNF-α, IL-6 and IL-10 in supernatants of ex vivo stimulated whole blood were determined batchwise by ELISA, according to the manufacturer's instructions (R&D systems, Minneapolis, MN, USA).

**HLA-DR expression**

RNA was isolated from blood collected in in Paxgene blood RNA tubes (Qiagen, Valencia, CA, USA). cDNA was synthesized from total RNA using the iScript cDNA Synthesis Kit (Bio-rad, Hercules, CA, USA). Subsequent qPCR analysis was performed using TaqMan gene expression assays (Bio-rad, Hercules, CA, USA) for the housekeeping gene PPIB (#Hs00168719_m1) and HLA-DR (#Hs00219575_m1) on a CFX96 Real-Time PCR Detection system (Bio-Rad, Hercules, CA, USA). HLA-DR was expressed as fold change relative to PPIB.

**Statistical analysis**

Data are expressed as mean±SEM or median [range or interquartile range (IQR)], according to their distribution (determined using the Kolmogorov-Smirnov test). Unpaired Student's T-tests or Mann–Whitney U tests were used to assess differences between healthy controls and patient samples. Statistical analysis was performed using Graphpad Prism 5 software (Graphpad Software, La Jolla, USA). A p-value of <0.05 was considered statistically significant.

## Results and Discussion

Patient characteristics are described in table 1.

| | |
|---|---|
| Sex (male/female) | |
| - Male | n=1 |
| - Female | n=5 |
| Disease classification | |
| - MDS-RAEB2 | n=2 |
| - AML | n=4 |
| o FAB M1 | n=2 |
| o FAB M4 | n=2 |
| % blasts (median [range]) | 47.5 [18-77] |
| Age (median [range]), yrs | 55.5 [47-62] |
| Chemotherapy | |
| - Cytarabin (200 mg/m$^2$) | n=6 |
| - Idarubicin (12 mg/m$^2$) | n=6 |
| - Clofarabin (10 mg/m$^2$) | n=2 |
| Relevant co-medication | |
| - Hydrea (day -3 to 8) | n=3 |
| - Allopurinol (day 0 to 8) | n=0 |

***Table 1 - Patient Characteristics***

As expected, leukocyte numbers gradually decreased after chemotherapy; at day 4 virtually no circulating leukocytes were present anymore (Figure 1A). Urate levels were within the reference range for our hospital (0.15-0.35 mmol/L) at all time-points (data not shown). Plasma LDH levels were slightly increased both before and after chemotherapy compared with the reference range for our hospital (<250 U/l, Figure 1B). Likewise, plasma levels of nDNA were significantly

increased in leukemia patients before chemotherapy, and these further increased 4-6 hours after start of chemotherapy (Figure 1C). These data are suggestive of cell damage and DAMP release related to both the haematological disease and chemotherapy. From day 4 onwards, plasma nDNA levels substantially decreased, probably related to the almost complete absence of leukocytes in the circulation. Levels of the specific DAMP mtDNA tended to increase following chemotherapy, but this did not reach statistical significance (p=0.07)(Figure 1D).

Before start of chemotherapy, plasma concentrations of some, but not all cytokines

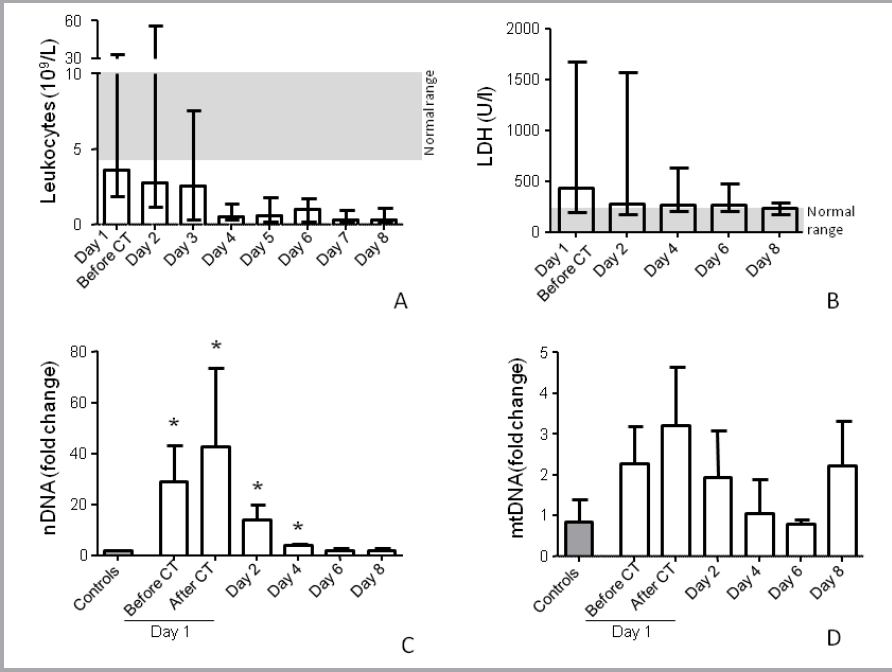


***Figure 1 - DAMP release in leukemia patients*** *Leukocyte counts (A) and plasma levels of LDH, nDNA and mtDNA (B, C, D). Data are depicted as median±interquartile range (Panel A+B) or mean±SEM (Panel C+D). 'Before CT' indicates before start chemotherapy. 'After CT' indicates 4-6 hours after start chemotherapy. *indicates p<0.05 compared with healthy controls.*

were above the detection limit (3.2 pg/ml) in leukemia patients: TNF-α: 13.1 pg/ml [7.8-22.8], IL-6: 22.1 pg/ml [4.0-39.2], IL-10: 11.6 pg/ml [8.2-35.9], and IL-1RA: 22.7 pg/ml [22.7-53.8]. Plasma cytokine levels did not change immediately following chemotherapy, indicating that no systemic inflammatory response was induced by the treatment (data not shown). LPS-induced *ex vivo* cytokine production until day 2 after chemotherapy was not significantly different compared with healthy controls (Figure 2, panels A, B, and C). From day 4 onwards, *ex vivo*

cytokine responses were below the detection limits of the assays, related to the very low numbers of leukocytes present in the circulation at the corresponding time points. *Ex vivo* cytokine production patterns were similar at all time points between patients who were treated with hydrea and those who were not. Finally, no differences in HLA-DR expression were observed between patients until day 2 post-chemotherapy and healthy controls (Figure 2D). No RNA could be isolated from blood at later time points, again related to the virtually complete absence of leukocytes in the circulation.

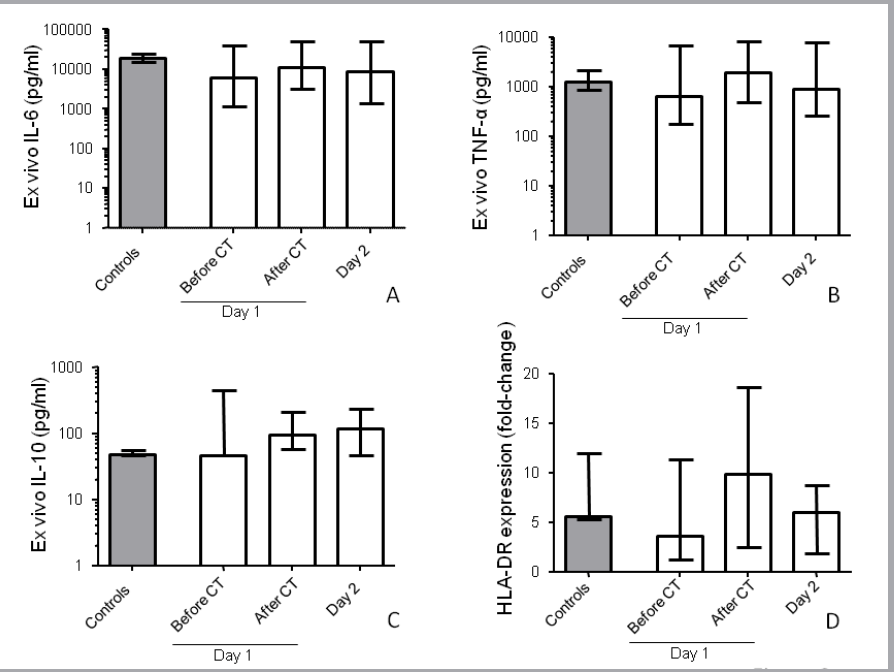


***Figure 2 – Leukocyte function in leukemia patients*** *Ex vivo cytokine response to LPS (A, B, C), and leukocyte HLA-DR mRNA expression (D). Data are depicted as median±IQR. 'Before CT' indicates before start chemotherapy. 'After CT' indicates 4-6 hours after start chemotherapy.*

Although defective immune function in leukemic cells has been described before [4,5], we demonstrate that cytokine production capacity and HLA-DR expression are not affected by DAMP release in leukemia patients treated with chemotherapeutic agents.

In conclusion, in the early phase following chemotherapy in leukemia patients, increased DAMP release does not induce immunoparalysis.

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# Chapter 5

## Plasma nuclear and mitochondrial levels, and markers of inflammation, shock, and organ damage in patients with septic shock

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*Shock*; in press

## Abstract

**Background** Plasma levels of the danger-associated molecular patterns (DAMPs) nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) have been shown to be related to sepsis mortality. However, the intermediate factors and/or mechanisms contributing to this relation are largely unknown. Our aim was to determine whether plasma levels of nDNA and mtDNA are related to the markers of inflammation, severity of shock, and organ damage in septic shock patients. Moreover, we investigated the relationship between plasma levels of nDNA/mtDNA and inflammatory cytokines during experimental human endotoxemia, a model of systemic inflammation in humans *in vivo* mimicking some of the hallmarks of early sepsis.

**Methods** Blood was sampled from the onset of septic shock until day 28 in 121 septic shock patients and from one hour before endotoxin administration until eight hours afterwards in 12 healthy volunteers. Plasma concentrations of 5 cytokines and circulating levels of nDNA and mtDNA were measured, and correlations with shock-related parameters and markers of organ damage were investigated.

**Results** In septic shock patients plasma cytokine concentrations, as well as nDNA and mtDNA levels, were increased at the onset of septic shock and remained elevated. During the first 5 days of septic shock, nDNA levels consistently correlated with plasma cytokine concentrations as well as with the shock-related parameter norepinephrine infusion rate and markers of organ damage (total bilirubin and creatinine). Experimental human endotoxemia also resulted in increased levels of plasma nDNA and mtDNA, but to a lesser extent than in septic shock patients. Furthermore, nDNA levels correlated with pro-inflammatory cytokines during endotoxemia.

**Conclusions** Our findings indicate a relationship between plasma nDNA levels and the inflammatory response. Furthermore, nDNA levels correlate with markers of shock and organ damage in septic shock patients. It remains to be determined whether nDNA is merely a marker or directly involved in the pathophysiology of septic shock.

## Introduction

Sepsis is the leading cause of death in intensive care units (ICUs), accounting for more deaths than breast cancer, lung cancer, and prostate cancer combined [1]. The development of sepsis is generally characterized by a simultaneously occurring hyperinflammatory and immunosuppressive reaction [2]. Pathogen associated molecular patterns (PAMPs) play an important role in the inflammatory response during sepsis, particularly in the hyperinflammatory reaction. However, danger associated molecular patterns (DAMPS) are released as well, contributing to initiation and/or propagation of inflammation [3, 4]. For instance, ongoing tissue damage and DAMP release have been demonstrated during lethal bacterial sepsis in primates, suggesting that both PAMP - and DAMP-related responses play a role in septic patients [4].

Nucleic acids released in the plasma during sepsis could serve as DAMPs. Both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) are released passively into the circulation after rupture or necrosis of cells [5], but active release has also been described [6-8]. mtDNA is of particular interest as a DAMP, as the endosymbiotic theory suggests that mitochondria originate from bacteria, and as such have their own DNA that strongly resembles bacterial DNA [9]. Both nDNA and mtDNA can bind to Pattern Recognition Receptors (PRRs) and thereby induce production of inflammatory cytokines [10]. Next to this possible role in initiation and/or propagation of inflammation, nucleic acids might also reflect the degree of shock and organ damage, and thus outcome in septic shock patients. Indeed, plasma nDNA and mtDNA levels have been shown to be related to sepsis mortality in several studies [11, 12]. However, the intermediate factors and/or mechanisms contributing to this relation have only sparsely been studied.

The aim of this study was to determine whether plasma nDNA or mtDNA levels are related to markers of inflammation, severity of shock, and organ damage, all of which are important hallmarks of sepsis that contribute to mortality. Moreover, we investigated the relationship between plasma levels of nDNA/mtDNA and inflammatory cytokines during experimental human endotoxemia, a model of systemic inflammation in humans *in vivo* mimicking some of the hallmarks of early sepsis [13, 14].

## Methods

### Study population

We performed a prospective observational study in 121 adult patients with newly developed septic shock between April 2012 and June 2014. Patients were included in the study according to conventional criteria for septic shock: a suspected infection and 2 or more SIRS criteria as well as requiring vasopressor therapy to maintain blood pressure [15, 16]. Patient characteristics are summarized in Table 1.

| | |
|---|---|
| Gender | Male n=71 (58.7%)<br>Female n=50 (41.3%) |
| Age (years, median[range]) | 66 [19-92] |
| APACHE II (median[range]) | 23 [11-45] |
| ICU length of stay (days, median [range]) | 7 [1-97] |
| Hospital length of stay (days, median [range]) | 26 [1-137] |
| 28-days-mortality | N=54 (44.6%) |
| Sepsis focus<br>- Urinary tract<br>- Lung<br>- Abdomen<br>- Venous/Arterial catheter<br>- Other | <br>n=12 (9.9%)<br>n=55 (45.5%)<br>n=37 (30.6%)<br>n=6 (5.0%)<br>n=26 (21.5%) |

***Table 1 – Patient Characteristics***

The study was carried out in the Netherlands in accordance with the applicable rules concerning the review of research ethics committees and informed consent. All patients or legal representatives were informed about the study details.

We also analyzed samples from 12 healthy male volunteers (median age 22 [range 19-27]) who participated in an experimental human endotoxemia study (NCT01835457 [17]. These samples were obtained from subjects of the control group who, besides LPS, did not receive any intervention. A detailed protocol of the endotoxemia study is described elsewhere [17]. Briefly, lipopolysaccharide (LPS, US Standard Reference Endotoxin *Escherichia Coli* O:113) was administered as an intravenous bolus injection at a dose of 2 ng/kg body weight in one minute at T = 0 hours. The subjects received 1.5 L 0.9% NaCl during one hour starting one hour before endotoxin infusion (pre-hydration) as part of our standard endotoxemia

protocol [18], followed by 150 ml/h until 6 hours after endotoxin infusion and 75 ml/h until the end of the experiment, 8 hours after infusion. The endotoxemia study was approved by the local ethics committee and written informed consent was obtained from all volunteers prior to screening and inclusion in the study. All study procedures were conducted in accordance with the declaration of Helsinki including current revisions and Good Clinical Practice guidelines.

**Sample and data collection**

Demographic data as well as clinical and laboratory parameters of patients (except for cytokine, nDNA, and mtDNA levels) were collected from electronic patient files. Blood from patients was sampled from the arterial catheter within 24 hours after diagnosis of septic shock (day 1). Further sampling took place as long as patients were admitted to the ICU on days 3, 5, 7, 9, 14, 21, and 28. Blood from healthy volunteers that underwent human endotoxemia was sampled from the arterial catheter 1 hour before LPS administration (T=-60), just before LPS administration (T=0), and 30, 60, 90, 120, 240, 360, and 480 minutes afterwards.

Ethylenediaminetetraacetic acid (EDTA) anticoagulated blood was centrifuged immediately after withdrawal at 1,600 x g at 4$^0$C for 10 minutes, after which plasma was stored at -80$^0$C until further analysis. Plasma for real time quantitative PCR (qPCR) analysis of nDNA and mtDNA levels was centrifuged again at 16,000 x g at 4$^0$C for 10 minutes to remove potential remaining cells and debris. The supernatant was stored at -80$^0$C until further analysis.

**Plasma cytokine concentrations**

Plasma concentrations of Tumor Necrosis Factor (TNF)-α and Interleukin (IL)-6, IL-8, IL-10, and IL-1 receptor antagonist (IL-1RA) were analyzed batchwise using a simultaneous Luminex assay according to the manufacturer's instructions (Milliplex; Millipore, Billerica, MA, USA).

**Nuclear and mitochondrial DNA quantification in plasma**

Double-centrifuged EDTA plasma was diluted 1:1 with phosphate buffered saline solution (PBS) after which DNA was isolated using the QIAamp DNA Blood Midi Kit (Qiagen, Valencia, CA, USA), using the 'Spin Protocol' as described by the manufacturer. Isolated DNA was stored at -20$^0$C until further analysis. qPCR was performed on a CFX96 Real-Time PCR Detection system (Bio-Rad Laboratories, Hercules, CA, USA). A primer pair specific for the GAPDH gene present in all nucleated cells of the body was used for quantification of nDNA levels: Forward 5'-AGCACCCCTGGCCAAGGTCA-3', Reverse 5''-CGGCAGGGAGGAGCCAGTCT-3'.

For quantification of mtDNA levels, the following primer pair specific for the mitochondrially encoded NADH dehydrogenase 1 (MT-ND1) gene was used: forward 5'-GCCCCAACGTTGTAGGCCCC-3' and reverse 5'AGCTAAGGTCGGGGCGGTGA-3'. Primers were obtained from Biolegio (Nijmegen, the Netherlands). The PCR reaction mixture consisted of 5 µl isolated DNA, 5.5 µl nuclease free water, 12.5 µl iQ SYBR Green PCR Master Mix (Bio-Rad Laboratories, Hercules, CA, USA) and 1 µl forward and reverse primer. Samples were analyzed in duplicate and a fresh aliquot of DNA isolated from blood obtained from a healthy volunteer was used in each plate as a calibrator (CV% of 1.9% (GAPDH) and 3.1% (mtDNA) between plates). Plasma nDNA and mtDNA quantities are expressed as fold change relative to the expression of the same gene in the calibrator sample.

**Statistical analysis**

Data are expressed as median [range/interquartile range (IQR)], according to their non-parametric distribution as determined using the Shapiro-Wilk test. Kruskal-Wallis with Dunn's post hoc tests were used to evaluate differences in plasma cytokine and DAMP levels between septic shock patients and healthy controls (samples obtained from healthy volunteers at baseline [T=-60, 1 hour before LPS administration]). Friedman with Dunn's post hoc tests were used to evaluate differences between baseline (T=-60) and other time-points during endotoxemia. Correlation coefficients were calculated using Spearman correlation. Statistical analysis was performed using Graphpad Prism 5 software (Graphpad Software, La Jolla, USA) and SPSS Statistics 20 (IBM, NY, USA). A two-sided p-value of $<0.05$ was considered statistically significant.

## Results

**Septic shock patients**

At virtually all time-points, plasma cytokine levels were higher in patients compared with those found in healthy controls before LPS administration (Figure 1, panels A-E), with highest levels observed at day 1.

At day 1, plasma nDNA levels were profoundly increased compared with healthy controls, and remained elevated during the next 28 days (Figure 2A). Although a similar pattern was observed for mtDNA, the increase was far less pronounced (Figure 2B). There were no differences in the nDNA/mtDNA ratio between controls and patients at any of the time-points (data not shown). Furthermore, plasma levels of nucleic acids at all time-points studied were similar between patients whose medical history included cancer and/or autoimmune diseases (n=57,

47%), conditions associated with increased levels of circulating nucleic acids [19, 20], and those in which it did not (data not shown).

Plasma nDNA levels correlated consistently with both pro- and anti-inflammatory cytokine concentrations in the initial phase of septic shock (Table 2). Moreover,

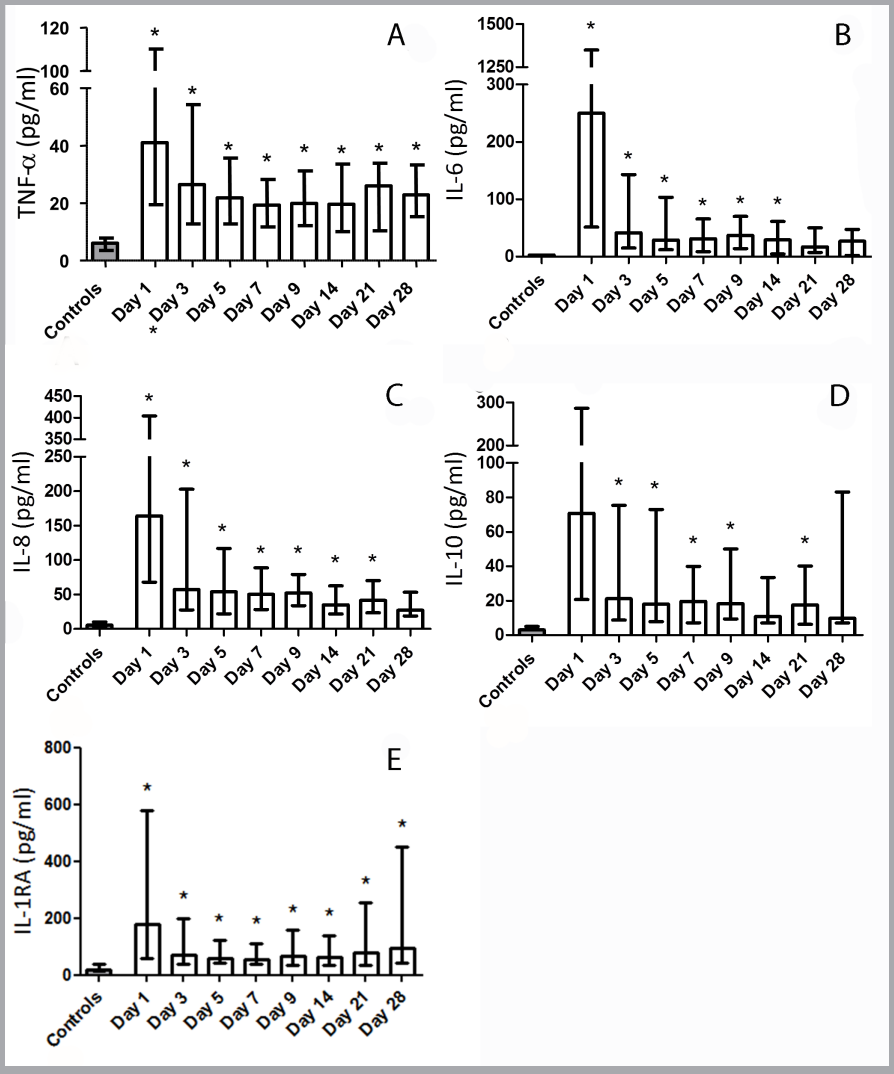


***Figure 1 – Plasma concentrations of inflammatory cytokines in septic shock*** *Plasma levels of pro- (A-C) and anti-inflammatory (D+E) cytokines in septic shock patients and healthy controls. Data are expressed as median±IQR. Day 1, n=109; Day 3, n=87; Day 5, n=61; Day 7, n=46; Day 9, n=38; Day 14, n=28; Day 21, n=21; Day 28, n=13. * indicates p<0.05 compared with controls.*

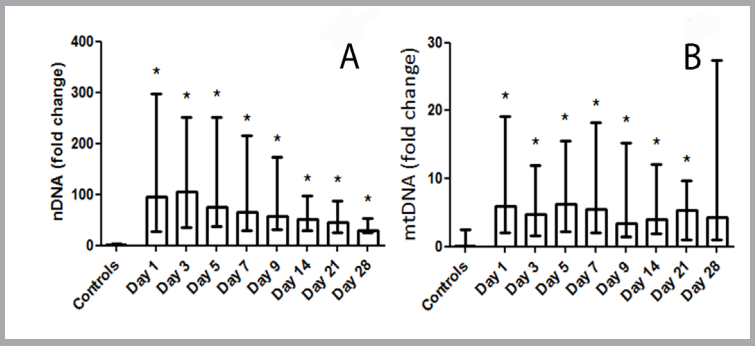


***Figure 2 – Plasma concentrations of DAMPs in septic shock*** *Plasma levels of DAMPs nDNA (panel A) and mtDNA (panel B) in septic shock patients and healthy controls. Data are expressed as median±IQR. Day 1, n=121; Day 3, n=92; Day 5, n=68; Day 7, n=50; Day 9, n=41; Day 14, n=29; Day 21, n=20; Day 28, n=16. * indicates p<0.05 compared with controls.*

plasma nDNA levels correlated with the shock-related marker norepinephrine dose as well as with creatinine and total bilirubin (markers of kidney and liver damage, respectively, Table 2). Although plasma mtDNA concentrations also showed a few statistically significant correlations with some of these parameters, no consistent effects were observed (Table 2).

**Healthy volunteers during human endotoxemia**

Following LPS administration, plasma levels of both pro- and anti-inflammatory cytokines profoundly increased (Figure 3).

nDNA levels were significantly increased compared with baseline from T=90 minutes onwards (Figure 4A). Plasma mtDNA levels demonstrated a similar pattern, although a significant difference with baseline values was observed only at T=180 minutes (Figure 4B). There were no differences in the nDNA/mtDNA ratio over time (data not shown). Peak nDNA levels correlated with peak IL-6 levels (r=0.72, p=0.008), whereas a similar trend was observed for the other pro-inflammatory cytokines (TNF-α:r=0.52, p=0.08; IL-8: r=0.55, p=0.07). No relationship between peak levels of nDNA and anti-inflammatory cytokines was found (IL-10: r=0.02, p=0.93, IL-1RA: r=0.02, p=0.96). Finally, no correlations between peak levels of plasma mtDNA and any of the studied cytokines were found (TNF-α: r=-0.25, p=0.42; IL-6: r=-0.06, p=0.85; IL-8: r=-0.34, p=0.26; IL-10: r=-0.52, p=0.08; IL-1RA: r=-0.32, p=0.31).

| | | nDNA | | | mtDNA | | | Patients |
|---|---|---|---|---|---|---|---|---|
| Inflammation | **TNF-α** Pro-inflam. | Day 1 | r=0.34 | p<0.001 | Day 1 | r=0.08 | p=0.44 | n=109 |
| | | Day 3 | r=0.36 | p=0.001 | Day 3 | r=0.13 | p=0.23 | n=86 |
| | | Day 5 | r=0.21 | p=0.111 | Day 5 | r=0.12 | p=0.37 | n=61 |
| | **IL-6** Pro-inflam. | Day 1 | r=0.21 | p=0.03 | Day 1 | r=0.08 | p=0.41 | n=109 |
| | | Day 3 | r=0.21 | p=0.06 | Day 3 | r=-0.06 | p=0.59 | n=86 |
| | | Day 5 | r=-0.02 | p=0.91 | Day 5 | r=-0.09 | p=0.51 | n=61 |
| | **IL-8** Pro-inflam. | Day 1 | r=0.34 | p<0.001 | Day 1 | r=0.16 | p=0.11 | n=109 |
| | | Day 3 | r=0.37 | p=0.001 | Day 3 | r=0.03 | p=0.77 | n=86 |
| | | Day 5 | r=0.24 | p=0.63 | Day 5 | r=0.08 | p=0.56 | n=61 |
| | **IL-10** Anti-inflam. | Day 1 | r=0.30 | p=0.001 | Day 1 | r=0.08 | p=0.43 | n=109 |
| | | Day 3 | r=0.34 | p=0.002 | Day 3 | r=0.08 | p=0.50 | n=86 |
| | | Day 5 | r=0.27 | p=0.04 | Day 5 | r=0.16 | p=0.22 | n=61 |
| | **IL-1RA** Anti-inflam. | Day 1 | r=0.38 | p<0.001 | Day 1 | r=0.20 | p=0.045 | n=109 |
| | | Day 3 | r=0.41 | p<0.001 | Day 3 | r=0.20 | p=0.08 | n=86 |
| | | Day 5 | r=0.25 | p=0.06 | Day 5 | r=0.21 | p=0.11 | n=61 |
| | **Leukocyte count** | Day 1 | r=0.10 | p=0.29 | Day 1 | r=0.53 | p=0.57 | n=119 |
| | | Day 3 | r=0.42 | p<0.001 | Day 3 | r=0.13 | p=0.24 | n=91 |
| | | Day 5 | r=0.32 | p=0.01 | Day 5 | r=0.31 | p=0.02 | n=60 |
| Shock | **Norepinephrine dose** | Day 1 | r=0.40 | p<0.001 | Day 1 | r=0.31 | p=0.001 | n=117 |
| | | Day 3 | r=0.30 | p=0.004 | Day 3 | r=0.14 | p=0.19 | n=94 |
| | | Day 5 | r=0.19 | p=0.13 | Day 5 | r=0.14 | p=0.28 | n=62 |
| | **MAP** | Day 1 | r=-0.08 | p=0.35 | Day 1 | r=-0.19 | p=0.04 | n=121 |
| | | Day 3 | r=-0.08 | p=0.45 | Day 3 | r=-0.12 | p=0.24 | n=92 |
| | | Day 5 | r=-0.10 | p=0.42 | Day 5 | r=-0.12 | p=0.33 | n=68 |
| Organ damage | **Creatinine** Kidney | Day 1 | r=0.28 | p=0.002 | Day 1 | r=0.14 | p=0.14 | n=120 |
| | | Day 3 | r=0.30 | p=0.006 | Day 3 | r=0.12 | p=0.13 | n=85 |
| | | Day 5 | r=0.29 | p=0.03 | Day 5 | r=0.11 | p=0.39 | n=59 |
| | **Total bilirubin** Liver | Day 1 | r=0.36 | p=0.003 | Day 1 | r=0.21 | p=0.10 | n=63 |
| | | Day 3 | r=0.32 | p=0.11 | Day 3 | r=0.04 | p=0.86 | n=26 |
| | | Day 5 | r=0.57 | p=0.05 | Day 5 | r=0.18 | p=0.56 | n=12 |

***Table 2 – Correlations between DAMPs and markers of inflammation, organ damage, and shock in patients with septic shock*** *Correlation coefficients were calculated between plasma levels of DAMPs and the various markers on corresponding days. Significant correlations are highlighted in grey. Cytokine and nucleic acid levels are depicted in Figures 1 and 2. On days 1 ,3, and 5, median [IQR] leukocyte counts were 14.0 [8.1-21.2], 11.4 [7.1-19.5], and 12.4 [7.6-17.2] X $10^9$/L, norepinephrine dose was 0.19 [0.10-0.50], 0.17 [0.10-0.30], and 0.1 [0.06-0.40] ug/kg/min, MAP was 74 [67-80], 77 [72-85], and 78 [71-88] mmHg, creatinine was 123 [74-230], 107 [66-227], and 104 [69-196] µmol/L, and bilirubin was 19 [12-35], 26 [13-86], and 26 [16-44] µmol/L.*

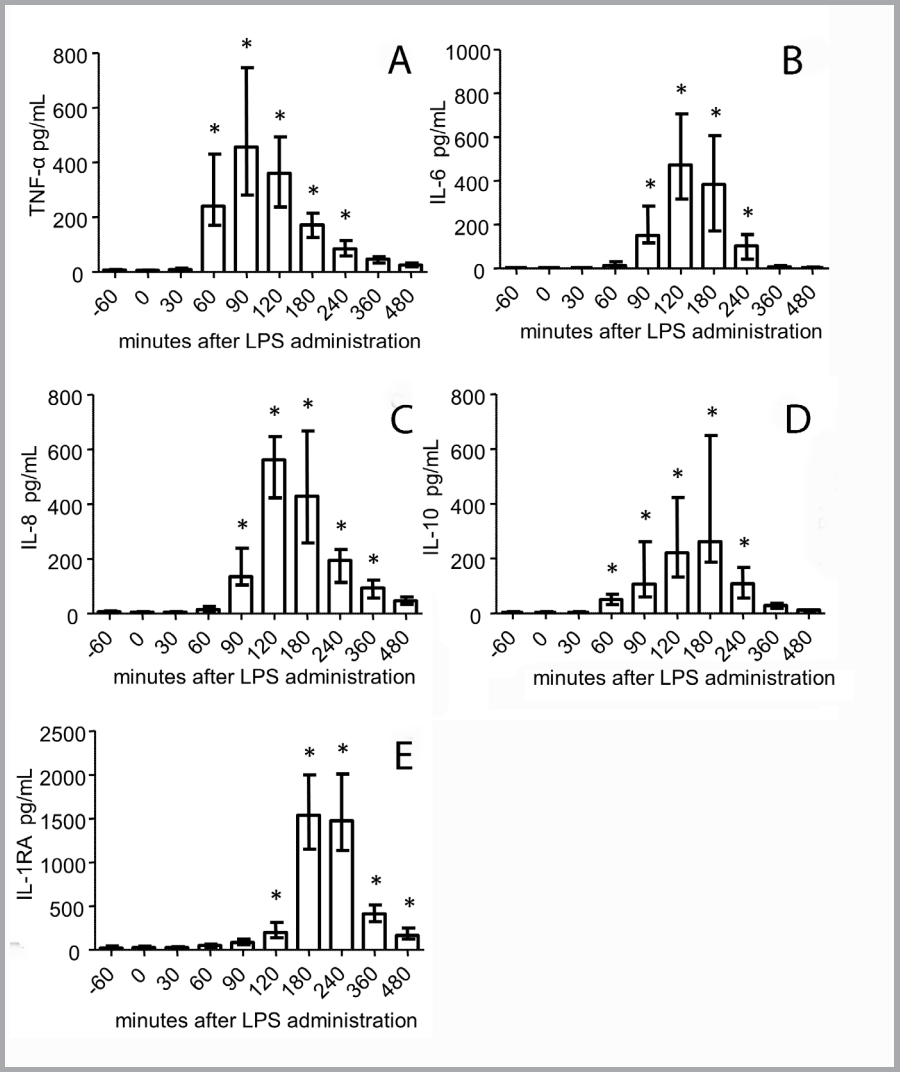


***Figure 3 – Plasma concentrations of inflammatory cytokines during experimental human endotoxemia*** *Plasma levels of pro- (A-C) and anti-inflammatory (D+E) cytokines in healthy volunteers during endotoxemia. Data are expressed as median±IQR. * indicates p<0.05 compared with time-point T=-60.*

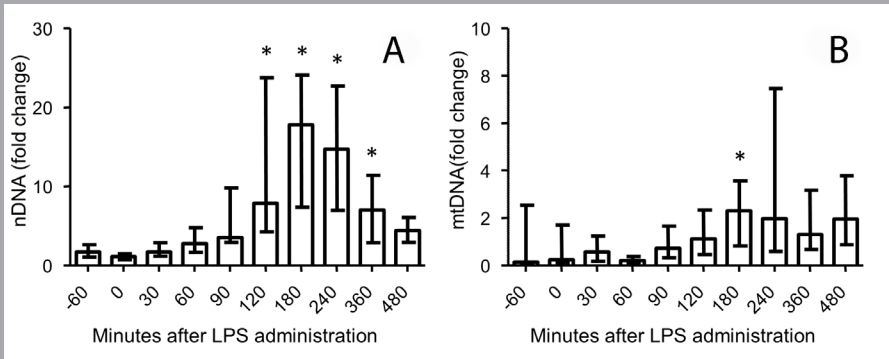


***Figure 4 – Plasma concentrations of DAMPs during endotoxemia*** *Plasma levels of DAMPs nDNA (panel A) and mtDNA (panel B) in healthy volunteers during endotoxemia. Data are expressed as median±IQR. * indicates p<0.05 compared with time-point T=-60.*



## Discussion

Herein, we demonstrate that plasma levels of nDNA and mtDNA are increased in septic shock patients. Furthermore, plasma levels of nDNA, but not mtDNA, are related to markers of inflammation, shock, and organ damage in these patients. Similar findings were obtained during human endotoxemia: LPS administration in healthy volunteers results in increased plasma levels of nDNA and mtDNA, although healthy volunteers exhibit lower peak levels compared with septic shock patients. Moreover, peak levels of nDNA, but not mtDNA, are related with the endotoxin-induced pro-inflammatory cytokine response.

It is generally assumed that nucleic acids are released after rupture or necrosis of cells [5]. However, our results obtained in the relatively mild human endotoxemia model demonstrate that a systemic inflammatory response, which is not expected to cause significant cell rupture or necrosis, also results in release of nucleic acids. These data suggests that inflammation results in active release of nucleic acids. Such active release, both spontaneous and during cell death, has been described previously, although the exact mechanisms remain to be elucidated [6-8]. Interestingly, plasma cytokine levels were higher in healthy volunteers during endotoxemia compared with septic shock patients, while levels of nucleic acids were much lower. While the pathogenesis of septic shock cannot be directly compared with experimental human endotoxemia, these differences suggest that in septic shock patients nucleic acids are released both actively as a result of

the inflammatory response and passively as a result of cell damage.

Our data further show that, compared with nDNA, the fold increase in plasma levels of mtDNA in sepsis patients compared with healthy controls as well as the fold increase observed in healthy volunteers following LPS administration is relatively low. Furthermore, mtDNA levels showed neither a relationship with inflammatory parameters in both patients and healthy volunteers, nor with clinical parameters in patients. These findings suggest that the role of mtDNA during sepsis and/or systemic inflammation is limited. nDNA might exert immunological effects, possibly via binding of PRRs, e.g. RIG-I (retinoic acid-inducible gene-I) or DAI (DNA-dependent activator of IFN-regulatory factors), both cytosolic receptors for double-stranded DNA [10]. In turn, activation of this inflammatory cascade may result in aggravation of shock and organ damage. However, the fact that higher levels of nDNA were found in septic patients than in healthy volunteers during endotoxemia, while concentrations of inflammatory cytokines were much lower might indicate that the role of nDNA in initiation and/or propagation of the inflammatory response is limited as well. This could be due to the fact that DAMP-properties of nucleic acids may be dependent on the release of other DAMPs, e.g. HMGB1, and that nucleic acids can also affect activity of other DAMPs [21]. PRRs recognizing nucleic acids are generally present intracellularly, and internalization necessary for receptor-binding is stimulated by other DAMPs [21]. These interactions underline the fact that multiple complex processes take place simultaneously in critically ill patients, making it difficult to distinguish causality from epiphenomena and unravel underlying mechanisms in observational studies.

This study has several limitations we want to acknowledge. First, the control group consist of young males, whereas the patient population comprises both males and females in a wide age range. With regard to gender, we investigated differences between male and female septic patients for all parameters described in this study and only found slightly, but statistically significant, higher levels of IL-6 and IL-8 on day 3 in women. No differences on other days were found. Therefore it is very unlikely that the presence of both men and women in our patient population relevantly biased our results. Concerning age, only 4 patients of our patient population fell within the age range of the healthy volunteers studied. Therefore, we could not analyze differences between young (i.e. those with comparable ages as the healthy volunteers) and old patients.
Second, the observational nature of our study does not permit us to draw definite

conclusions as to whether there is a causative relation between nDNA levels, inflammation, shock, and organ damage. However, in a recent murine study, administration of DNAses (to remove free nucleic acids from the circulation) resulted in decreased coagulation and inflammation, suppression of organ damage, and improved outcome in a cecal ligation and puncture sepsis model [22]. These findings suggest a detrimental role of nucleic acids in sepsis.
Third and finally, although statistically significant, the correlations between nDNA and markers of inflammation, shock, kidney, and liver damage in septic patients were relatively weak. Therefore caution should be taken when interpreting these results. The well-known high heterogeneity of septic shock patients might contribute to this.

In conclusion, plasma levels of nucleic acids mtDNA and especially nDNA are increased in septic shock patients and healthy volunteers during experimental human endotoxemia, and nDNA levels are related with inflammatory cytokines. This suggests that nucleic acids, particularly nDNA, are both actively and passively released; as a result of inflammation and cell damage, respectively. Furthermore, nDNA levels correlate with markers of shock and organ damage in septic shock patients. It remains to be determined whether nDNA is merely a marker or directly involved in the pathophysiology of septic shock.

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# Chapter 6

## Blueprints of signaling interactions between pattern recognition receptors: implications for the design of vaccine adjuvants

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## Abstract

**Introduction** Innate immunity activation largely depends on recognition of micro-organism structures by Pattern Recognition Receptors (PRRs). PRR downstream signaling results in production of pro- and anti-inflammatory cytokines and other mediators. Moreover, PRR engagement in antigen-presenting cells initiates the activation of adaptive immunity. Recent studies suggest that for the activation of innate immune responses and initiation of adaptive immunity, synergistic effects between two or more PRRs are necessary. No systematic analysis of the interaction between the major PRR pathways has been performed to date. In this study, a systematical analysis of the interactions between PRR signaling pathways was performed.

**Methods** PBMCs derived from ten healthy volunteers were stimulated with either a single, or a combination of two PRR ligands. Known ligands for the major PRR families were used: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and RigI-helicases. After 24h incubation, production of TNFα, IL-1β, IL-6, and IL-10 was measured in supernatants by ELISA. The consistency of the PRR interactions (both inhibitory and synergistic) between the various individuals was assessed.

**Results and discussion** A number of PRR-dependent signaling interactions were found to be consistent, both between individuals and with regard to multiple cytokines. The combinations of TLR-2 and NOD-2, TLR-5 and NOD-2, TLR-5 and TLR-3, and TLR-5 and TLR-9, acted as synergistic combinations. Surprisingly, inhibitory interactions between TLR-4 and TLR-2, TLR-4 and Dectin-1, TLR-2 and TLR-9 as well as TLR-3 and TLR-2, were observed. These consistent signaling interactions between PRR combinations may represent promising targets for immunomodulation and vaccine adjuvant development.

## Introduction

The first step in mounting an appropriate host defense to infection is the recognition of pathogens by the innate immune system. This recognition is mediated through so-called pattern recognition receptors (PRRs) expressed on the cell membrane of cells of the innate immune system [1, 2]. The recognition of pathogens by PRRs leads on the one hand to the activation of inflammation, or innate host defense, and on the other hand to the initiation of adaptive immunity, and eventually to immunological memory, as pursued in vaccination strategies.

PRRs recognize Pathogen-Associated Molecular Patterns (PAMPs), conserved motifs derived from a broad spectrum of pathogens, including fungi, bacteria, parasites, and viruses [1, 2]. Hence, these receptors can provide highly specific recognition of a vast range of microbes [3]. Recognition of a PAMP by a PRR results in the activation of the innate immune system via several intracellular signaling pathways that are described in more detail elsewhere [4–6]. Several major families of PRRs have been described to date, including the Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and RigI-helicases [7–9]. TLRs and CLRs are present on the cell membrane and in endosomes, while NLRs and RigI-helicases are intracellular microbial sensors [2, 10].



Important adaptor molecules of the TLR intracellular pathways are MyD88, TRIF, TRAM, and MAL/TIRAP [11]. Other adaptor molecules such as Syk and Raf-1 (in the case of CLRs) or Rip2/RICK (in the case of some NLRs such as NOD1/NOD2) are also involved in intracellular signaling [12, 13]. PRRs recruit one or more of these adaptor molecules in order to provide specific signaling [5]. Activation of signaling pathways ultimately leads to the production of pro- and/or anti-inflammatory cytokines, such as those mediated by the transcription factors nuclear factor κB (NF-κB) and activating factor 1 (AP1), that induce production of inflammatory cytokines and shape the subsequent adaptive immune response [7, 14, 15].

Previous studies have indicated that some PRRs are able to interact with each other, thereby modulating the magnitude and/or type of cytokine production with synergistic or inhibitory effects or both [4, 16–18]. However, whether these interactions are generally embedded in the innate immune system and biologically conserved between different individuals is not known. The identification of the most consistent PRR interactions would provide insight into the interplay of the signaling pathways that modulate cytokine responses in the majority of

the individuals in a population, with important consequences for the design of vaccine adjuvants. In this study, we investigated signaling interactions between several PRRs in order to identify biologically conserved signaling in cytokine responses of innate immune cells. Ultimately, this could lead to combinations of PRR agonists that can be used as a vaccine adjuvant.

## Materials and Methods

### Volunteers

Blood samples were collected from 10 healthy volunteers. After informed consent was obtained, blood was collected into 10-ml EDTA tubes (BD, Plymouth, United Kingdom).

### PBMC isolation and stimulation with PRR ligands

Peripheral blood mononuclear cells (PBMCs) were isolated as described previously [19]. Briefly, a PBMC fraction was obtained by differential centrifugation over Ficoll-Paque. PBMCs (5 × 105 cells per well) were incubated for 24 h at 37°C in round-bottom 96-well plates (Cellstar; Greiner Bio-one, Alphen a/d Rijn, the Netherlands) with a single ultrapure PRR ligand or a combination of two. RPMI 1640 Dutch modification culture medium (Sigma-Aldrich, Zwijndrecht, the Netherlands) was used, which was supplemented with 1% gentamicin, 1% l-glutamine, and 1% pyruvate (Life Technologies, Nieuwerkerk, the Netherlands). The concentrations of the different ultrapure ligands used are based on literature research and experience in our laboratory and are described in Table 1. These concentrations give a robust but nonmaximal response of most cytokines if used as a single ligand to stimulate PBMCs. This allows detection of increases in cytokine production upon combination with another ligand. Therefore, the concentrations used are suitable to study synergistic or inhibitory effects.

After stimulation, the plates were centrifuged (8 min, 1,700 rpm, room temperature [RT]) and the supernatants were collected and stored at −20°C until analysis. The stimulation experiments were performed in duplicate, and supernatants from duplicate wells were pooled for cytokine determination using enzyme-linked immunosorbent assays (ELISA). Each combination of ligands was used with PBMCs of 10 different volunteers.

### Enzyme-linked Immunosorbent Assay (ELISA)

Cytokine concentrations in culture supernatants were determined using commercially available ELISA kits for tumor necrosis alpha (TNF-α) and interleukin-1 beta (IL-1β) (R&D Systems, Abingdon, Oxfordshire, United Kingdom) and IL-10

| PRR | Ligand | Concentr. | Origin | Median Cytokine Production |
|---|---|---|---|---|
| **TLR2** | Pam3Cys | 10 µg/mL | EMC Microcollections, Tuebingen,Germany | IL-1β, 445 pg/ml; TNF-α, 270 pg/ml; IL-6, 5,462 pg/ml; IL-10, 145.4 pg/ml |
| **TLR3** | Poly I:C | 50 µg/mL | Invivogen, San Diego, CA | IL-1β, 39 pg/ml; TNF-α, 143.5 pg/ml; IL-6, 236.5 pg/ml; IL-10, 15.7 pg/ml |
| **TLR4** | E. coli LPS (O55,B5) | 1 ng/ml | Sigma-Aldrich, St. Louis, MO (further purified as described previously [20] | IL-1β, 525 pg/ml; TNF-α, 315.5 pg/ml; IL-6, 9,200 pg/ml; IL-10, 279 pg/ml |
| **TLR5** | Flagellin | 1 µg/mL | Invivogen, San Diego, CA | IL-1β, 247 pg/ml; TNF-α, 145.5 pg/ml; IL-6, 5,775 pg/ml; IL-10, 139.2 pg/ml |
| **TLR9** | CpG | 10 µg/mL | Invivogen, San Diego, CA | IL-1β, 39 pg/ml; TNF-α, 143.5 pg/ml; IL-6, 236.5 pg/ml; IL-10, 15.7 pg/ml |
| **NOD2** | Muramyldipeptide (MDP) | 10 µg/mL | Sigma-Aldrich, Buchs, Switzerland | IL-1β, 39 pg/ml; TNF-α, 78 pg/ml; IL-6, 121.2 pg/ml; IL-10, 7 pg/ml |
| **Dectin-1** | β-glucan | 10 µg/mL | Kindly provided by G. D. Brown, University of Aberdeen, United Kingdom | IL-1β, 39 pg/ml; TNF-α, 78 pg/ml; I L-6, 17.3 pg/ml; IL-10, 7 pg/ml |
| **Mannose receptor (MR)** | Mannan | 10 µg/mL | Sigma-Aldrich, Buchs, Switzerland | IL-1β, 39 pg/ml; TNF-α, 78 pg/ml; IL-6, 15.6 pg/ml; IL-10, 4.7 pg/ml |

***Table 1 – Ultrapure PRR ligands used in stimulation experiments with PBMCs***

and IL-6 (Pelikine Compact; Sanquin Reagents, Amsterdam, the Netherlands). The measurements were performed according to the manufacturer's instructions.

**Calculations and statistical analysis**

The criteria according to which interactions between two specific signaling pathways downstream of activated PRRs were considered synergistic or inhibitory were defined before the start of the experiments. A "synergistic effect" was defined as a cytokine response to a combination of ligands that was at least 1.5-fold higher than the sum of the cytokine responses induced by each of the individual ligands. An "inhibitory effect" was defined as a cytokine response to a combination of ligands that was less than or equal to 0.75-fold of the sum of the cytokine responses to each of the individual ligands and lower than the cytokine response to a single ligand or to both of the ligands. The definitions of both synergy and inhibition are further explained in Figure 1. All other patterns were designated "no effect/additive effect."

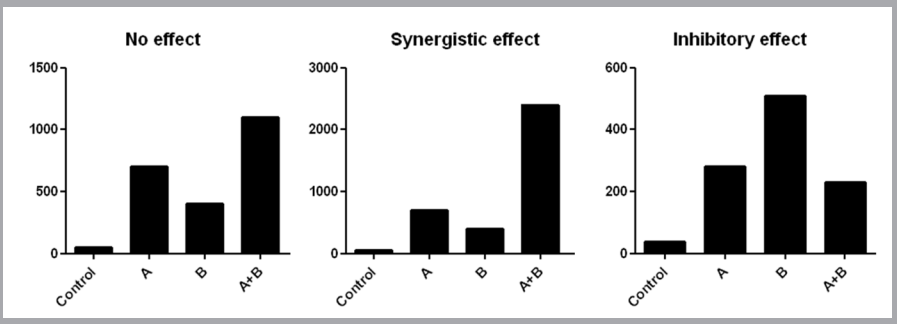


***Figure 1 – Interaction classifications On the y-axis, production of a cytokine is depicted.*** *On the x-axis the ligands (A and B) used to stimulate the PBMCs are depicted; PBMCs were stimulated either with a single ligand or a combination of two PRR ligands. To identify a synergistic effect, the cytokine production after stimulation with both ligands combined should be higher than the value of both single effects together. An inhibitory effect was defined as a cytokine response to a combination of ligands that was maximally 0.75-fold the sum of the cytokine response to either of the individual ligands and lower than the cytokine response to a single or both of the ligands. A cytokine production of the combination of stimuli higher than in each of the single stimulations, but not higher than the sum of both is considered additive and is thus classified as "no effect/additive effect"*

An effect was considered present (biologically conserved) in the majority of the healthy volunteers tested if a type of interaction was demonstrated in at least 7 of the 10 subjects. If there was more variation between subjects, it was defined as a "variable effect."
The biologically conserved interactions (depicted in red and blue in Figure 2) were analyzed statistically using Wilcoxon matched-pair tests. Sums of cytokine concentrations after single-ligand stimulations were compared to cytokine concentrations after combined stimulations. Differences were considered significant if $P < 0.05$.

## Results

**Interaction studies**
The interactions between different PRR ligands are depicted in Figure 2. An inhibitory effect for comparisons of the production of IL-1β, IL-6, and TNF-α between TLR-4 and Dectin-1 (significant for IL-1β and IL-6) as well as TLR-3 and TLR-2 was found for IL-1β and IL-6. Similar effects were found for combinations of TLR-4 and TLR-2 or TLR-2 and TLR-9 induction of IL-1β production.



The combination of TLR-5 and TLR-9 resulted in synergistic effects for IL-1β and IL-6 production. Furthermore, synergistic effects on IL-1β, IL-6, and IL-10 production were observed for the combinations TLR-2 and NOD-2 (significant for IL-10), TLR-5 and NOD-2 (significant for IL-10), and TLR-5 and TLR-3 (significant for IL-1β). A synergistic effect was also found for IL-10 and IL-6 production after stimulation of a combination of TLR-3 and NOD-2 or NOD-2 and Dectin-1 (significant for IL-10). Stimulation of TLR-5 and Dectin-1 resulted in a synergistic effect on IL-10 production, but other cytokines showed only variable effects.

Of interest, the synergistic effects of TLR-3 and TLR-5 and of TLR-5 and TLR-9 stimulation mentioned above were observed in at least 9 of the healthy volunteers for IL-1β (significant) and IL-10 production and for IL-1β production (significant), respectively (Figure 3).
Several PRRs did not interact with others. In particular, mannose-receptor-dependent induction of cytokine production seemed independent of activation of other PRRs.

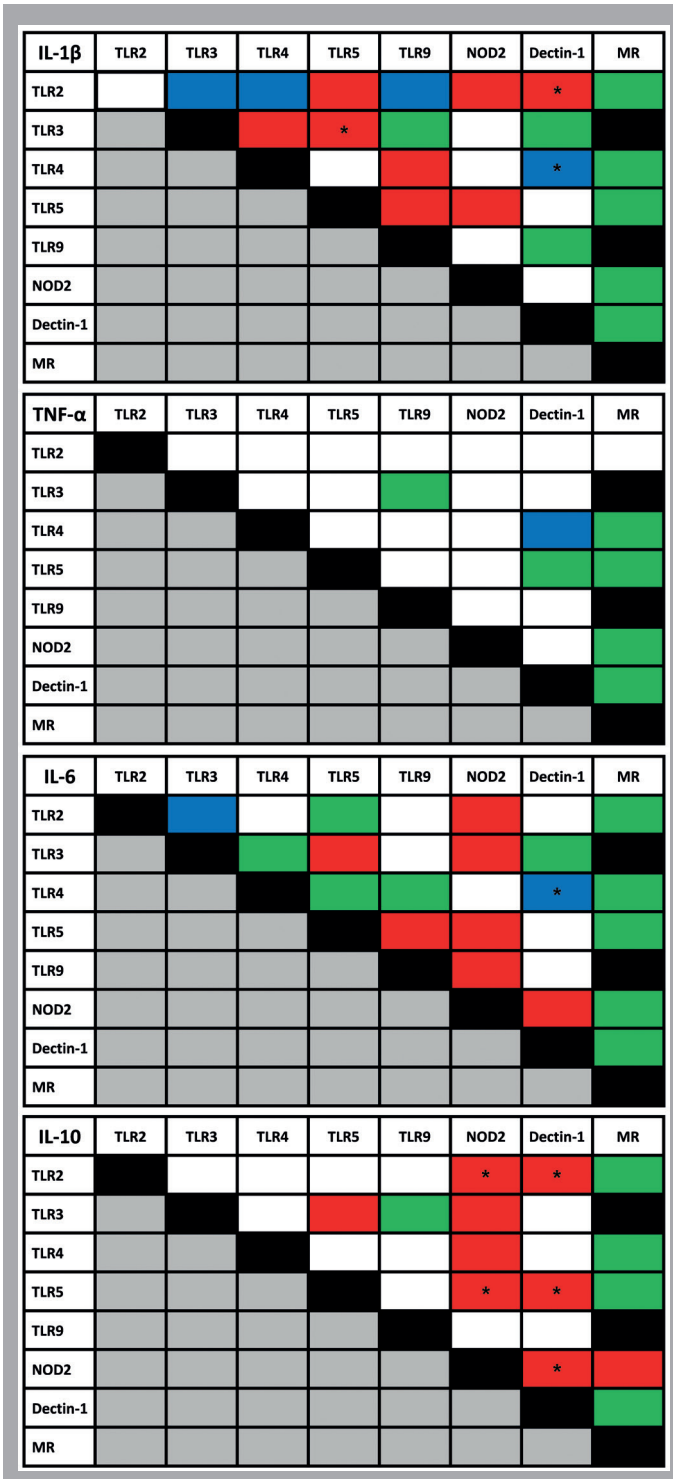


***Figure 2 – Overview interactions PRRs*** *Color indicates type of interaction in at least seven out of 10 of the healthy volunteers. ELISA on culture supernatants after 24 hours of stimulation at 37$^0$C with combinations of ligands as specified in table 1. * indicates p<0.05 (Wilcoxon matched pairs test, comparison of cytokine production upon stimulation with both ligands with sum of cytokine production upon stimulation with each of the ligands separately).*

*Red = Synergistic effect*
*Green = No effect/additive effect*
*Blue = Inhibitory effect*
*White = Variable effect*
*Black = Experiment not performed*

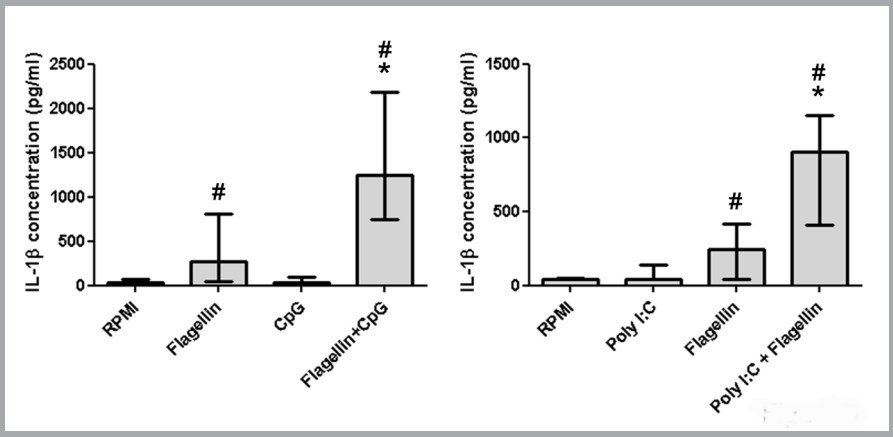


***Figure 3 – Flagellin interactions*** *PBMCs were stimulated for 24 hours with the indicated ligands or combinations of ligands and cytokines were measured in the supernatants by ELISA (n=10 volunteers) The bars indicate a synergistic interaction of flagellin with CpG or Poly I:C for the production of IL-1β. Data are expressed as medians with interquartile range.*
** = significantly different (p<0.05) compared with stimulation with single ligands and the sum of both single ligands, # = significantly different (p<0.05) compared with no ligand (RPMI) (Wilcoxon matched pairs test).*



**TLR-2/TLR-4 interaction**

The results of our study indicate an inhibitory effect between TLR-2 and TLR-4 for the production of IL-1β. This is in contrast with previous studies [18, 21–25]. In order to study the interaction between TLR-2 and TLR-4 in more detail, a dose-response experiment with lipopolysaccharide (LPS) and Pam3Cys was performed. Increasing concentrations of LPS or Pam3Cys resulted in increased IL-1β and TNF-α production, but the combination of the two ligands showed an inhibitory effect (Figure 4), confirming our initial results.

**Interindividual variation**

In this study, the interactions between PRRs were highly variable between subjects. To illustrate the interindividual variation in PRR signaling interactions, the interaction between NOD-2 and TLR-4 for the production of TNF-α in all 10 healthy volunteers is depicted in Figure 5. In most volunteers, muramyl dipeptide (MDP)-induced TNF-α production was around 100 to 200 pg/ml. LPS stimulation generally resulted in higher levels of TNF-α than MDP stimulation. The response to the combination of the two ligands was highly variable between subjects, and no effects/additive effects, inhibitory effects, or synergistic effects on TNF-α production were observed in the different volunteers.

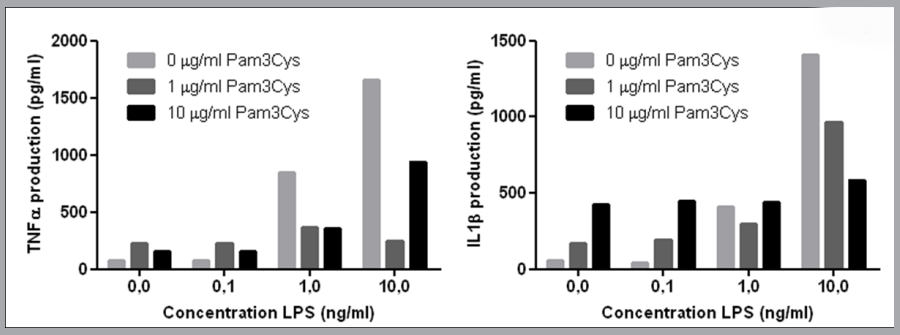


***Figure 4 – Dose-response LPS + Pam3Cys*** *PBMCs were stimulated for 24 hours with the indicated ligands or combinations of ligands and cytokines were measured in the supernatants by ELISA (n=2 volunteers). The bars indicate a different concentration of Pam3Cys, while an increasing concentration of LPS is depicted on the x-axis. Increasing concentrations of LPS and Pam3Cys resulted in increased IL-1β and TNFα production for single ligands, but no synergistic production of either IL-1β or TNF-α was observed if LPS and Pam3Cys were combined. Data are depicted as medians.*

## Discussion

The aims of this study were to systematically investigate the interactions between the best-known PRR pathways on the one hand, and to assess the consistency of these effects in various individuals on the other hand. Knowledge on the consistency of the synergistic combinations of PRR pathways could be used to develop novel vaccine adjuvants that would exert their boosting effect during vaccination in the vast majority of the individuals in a population. This study resulted in the identification of several combinations of PRR ligands that have this potential.

A remarkable, yet not entirely unexpected finding of this study is the large variability between healthy individuals. This variation can be attributed to many factors. One of these is the genetic background of the individuals tested. There are many established variations in genes that are known to influence the function and downstream effects of PRRs, which could naturally influence the cytokine production after stimulation with PRR ligands [26, 27]. This interindividual variation has important implications with regard to the design of immunotherapeutic approaches based on PRR-mediated effects. For the development of vaccine adjuvants based on PRR-ligand combinations, only those combinations that result in synergistic cytokine production in the majority of the population are

likely to be of therapeutic value.

In this respect, in the present report we identify several consistent PRR interactions that were present in the majority of individuals tested. The combinations of ligands most consistently associated with synergistic effects were NOD-2 and TLR-2, NOD-2 and TLR-5, TLR-3 and TLR-5, and TLR-5 and TLR-9. In particular, the interaction of TLR-5 with TLR-3 and TLR-9 was remarkably consistent, since it was present in at least 9 of the studied volunteers for all the cytokines studied. These combinations could therefore represent candidates for the development of novel vaccine adjuvants. In the pursuit of identifying novel vaccine adjuvants to activate the immune system, synergy for IL-10 production could be disadvantageous, since IL-10 is known to have potent anti-inflammatory effects [28]. In that respect, the combination of TLR-5 and TLR-9 appears also promising, because IL-1β, but not IL-10, is produced in a synergistic manner after engagement of these receptors.

In addition to the synergistic effects of some PRR combinations as detailed above, a number of biologically conserved inhibitory combinations were also documented: TLR-4 and Dectin-1, TLR-3 and TLR-2, TLR-2 and TLR-9, and TLR-4 and TLR-2 result in impaired cytokine production. These findings are surprising, as they contradict a number of studies published in the literature suggesting additive or even synergistic effects of ligands for these receptors. This is particularly true for the TLR-4-TLR-2 costimulation [18, 21–25]. We have performed additional experiments to assess the validity of our observation, and a dose-response experiment with LPS and Pam3Cys confirmed our initial findings. The most likely explanation for the difference between our findings and those in previous studies is that we have used primary human PBMCs, and we assessed the interindividual consistency of these stimulations, while previous studies have mostly used either mouse cells or human cell lines [18, 21–25].

Next to these observations regarding the consistency of the biological effect of PRR-ligand combinations, additional important observations can be made. First, the interaction patterns differed substantially between the four measured cytokines. There are relatively more combinations of ligands that exhibit synergism in the production of IL-10, IL-1β, and IL-6 than of TNF-α. These differences could theoretically be due to the existence of (partly) separate signaling pathways for these cytokines. It is also conceivable that a pathway leading to production of one cytokine is more biologically conserved than that leading to another cytokine.
Second, it appears that there are several main "intracellular highways" which

comprise PRRs that preferentially interact with each other. Multiple PRRs within one highway effectively influence each other, while PRRs in different highways do not appear to interact. The mannose receptor, for example, appears not to interact with the signaling pathways of other PRRs and thus can be regarded as an isolated highway. Alternatively, our results of mannose-receptor stimulation could have been influenced by the very low number of mannose receptors present on the surface of monocytes [29]. TLR-4 signaling, although to a lesser extent, also appears to represent an isolated pathway: when PBMCs are stimulated with LPS, a relatively high number of combinations of ligands result in a noninteractive/additive cytokine production. In addition to the mannose-receptor and TLR-4 pathways, we can also distinguish a group of receptors which appear to show complex interactions with each other, that may be considered a "TLR-2-Dectin-1-NOD-2-TLR-3-TLR-5 highway." These receptors seem to interact with each other very effectively. TLR-9 also seems to be involved in interactions with the intracellular pathways from this group of receptors, but specifically for the induction of IL-1β and IL-6 production, and to a lesser extent for IL-10 and TNF-α production. In particular, cytokine responses through dectin-1 signaling are dependent on these interactions, since stimulation with β-glucan alone results in very limited cytokine production. Therefore, it is possible that dectin-1 signaling is dedicated to the regulation and enforcement of the effect of other PRRs, rather than the activation of the immune system by itself.

Several limitations apply to our study. First of all, the molecular mechanisms behind the interactions described in the present report have not been explored and should be evaluated in future studies. These interactions could be the result of intracellular interactions between signaling pathways, but they could also be regulated via release of soluble regulatory factors such as cytokines. Furthermore, altered expression of receptors by (combinations of) ligands could also confound true interaction effects between these receptors. Future studies that focus on the use of the herein-identified combinations of ligands as vaccine adjuvants should include specific cytokines with specific properties (e.g., IP-10 or interferon [IFN] for the TRIF pathway, or potential indicators for Th1 responses) and detailed data on dendritic cells and T cell responses. Finally, multiple time points could be included in future studies; in particular, information on early responses (4 to 6 h) would be helpful.

In conclusion, the present report provides new information with regard to the pathway interactions downstream of PRR signaling that lead to the induction

of innate host responses and initiate the adaptive immunity. Unraveling these signaling cascades not only is vital for the understanding of the innate immune system in general, but also could help to identify new targets for immunomodulation [3, 30]. Furthermore, the consistency of the biological synergistic interactions between several combinations of PRRs may be important to the field of vaccine adjuvant research [18]. Robust activation of the innate immune system, subsequently evoking an adaptive immune response involving memory T cells, is vital for the effectiveness of a vaccine. Based on the present data, specific combinations such as TLR-5/TLR-3 and TLR-5/TLR-9 appear particularly promising, as their synergistic interactions were biologically conserved in the majority of the individuals tested in the present study population. Therefore, the potential of these combinations of ligands as vaccine adjuvants should be further explored to improve vaccination strategies. Nevertheless, care should be taken that certain ligand combinations do not result in a too-pronounced immune response resulting in tissue damage and organ failure. Hence, future studies should take into account the balance between the immune response required for immunization and potential harmful effects.

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# Part II

## DAMPs and ventilator-induced inflammation

# Chapter 7

## Mitochondrial DNA and TLR9 signaling is not involved in mechanical ventilation-induced inflammation

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*Submitted*

## Abstract

**Background** Exogenous administration of mitochondrial DNA (mtDNA) causes inflammatory lung injury in a Toll-like receptor (TLR) 9-dependent manner. We investigated whether mechanical ventilation results in endogenous release of mtDNA and if TLR9 plays a role in the pulmonary inflammatory response induced by mechanical ventilation.

**Methods** Wild-type and TLR9$^{-/-}$ C57bl/6 mice were ventilated with low (8 ml/kg) and high (32 ml/kg) tidal volumes for four hours. Levels of nuclear (nDNA) and mtDNA in bronchoalveolar lavage fluid as well as pulmonary concentrations of KC, IL-1β, and IL-6 were determined.

**Results** Cytokine and nDNA, but not mtDNA, levels were increased following mechanical ventilation with both tidal volumes. Cytokine concentrations were similar between wild-type and TLR9$^{-/-}$ mice.

**Conclusions** Mechanical ventilation does not result in the release of mtDNA and TLR9 is not involved in mechanical ventilation-induced inflammation.

## Introduction

Mechanical ventilation (MV) is an essential part of perioperative and intensive care medicine. However, MV can induce an inflammatory response in the lungs which compromises pulmonary function, and can result in organ dysfunction [1, 2]. Danger Associated Molecular Patterns (DAMPs) originating from injured or dead cells, or from the extracellular matrix are implicated in the pathogenesis of MV-induced inflammation and lung injury [3, 4]. Possible mechanisms behind the release of DAMPs in this setting include stretch due to cyclic opening and closing of alveoli and/or direct tissue damage resulting from volu-or barotrauma. Mitochondrial DNA (mtDNA) is a DAMP that causes inflammatory lung injury in a Toll-like receptor (TLR) 9-dependent manner when administered exogenously in the lungs or circulation of rats and mice [5, 6]. Furthermore, acid aspiration in mice results in profound increase in mtDNA levels in bronchoalveolar lavage (BAL) fluid [7]. In the present study, we investigated whether MV with low or high tidal volumes results in endogenous release of mtDNA and if TLR9 plays a role in the MV-induced pulmonary inflammatory response.

## Methods



All experiments were approved by the Regional Animal Ethics Committee in Nijmegen. The study has been conducted in a manner that does not inflict unnecessary pain or discomfort upon the animals, as outlined by the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals (1996), prepared by the National Academy of Sciences' Institute for Laboratory Animal Research.

### Animals

Age-matched male wild-type C57Bl/6 mice and TLR9-deficient (TLR9$^{-/-}$) mice with a C57Bl/6 background, aged 8 to 14 weeks, weighing 25±4 g were used in this study (n=8 per group). Wild-type mice were purchased from Charles River laboratories (Leiden, the Netherlands), TLR9$^{-/-}$ mice were obtained from S. Akira (Department of Host Defense, Osaka University, Osaka, Amsterdam). The mice were housed in a light- and temperature-controlled room under specific pathogen-free (SPF) conditions. Standard pelleted chow (SSNIFF Spezialdiäten GmbH, Soest, Germany) and drinking water were available *ad libitum*. Eight mice per group were used.

**Experimental procedures**

Mice were anaesthetized using an intraperitoneal injection of 7.5 µl per gram body weight of KMA mix (25.5 mg/ml ketamine, 40 µg/ml medetomidine, 0.1 mg/ml atropine in saline). Subsequently, the animals were intubated and mechanically ventilation was performed using a tidal volume of 8 mL/kg at a frequency of 170 breaths per minute using a MiniVent ventilator (Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany) or a tidal volume of 32 mL/kg at a frequency of 40 breaths per minute using a Ugo Basile UB7025 ventilator (Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany). Lipopolysaccharide (LPS) was measured in the mechanical ventilation circuit by the Limulus Amebocyte Lysate assay (Cambrex Bio Science, Walkersville, MD, USA; detection limit: 0.06 IU/ml) to rule out LPS-induced pulmonary inflammation. All animals received 1.5 cm H2O positive end-expiratory pressure (PEEP), and fraction of inspired oxygen was set to 0.4. In order to maintain anaesthesia, boluses of 5.0 µl per gram body weight maintenance KMA mix (3.6 mg/ml ketamine, 4 µg/ml medetomidine, 7.5 µg/ml atropine in saline) were given every 30 min via an intraperitoneally placed catheter. Rectal temperature was monitored continuously and maintained between 36.0°C and 37.5°C using a heating pad. After the four-hour ventilation period, mice were sacrificed by exsanguination. Control mice were anaesthetized, but not ventilated, and sacrificed shortly after induction of anaesthesia.

**Sample collection and analysis**

In wild-type mice, bronchoalveolar lavage (BAL) was performed immediately before sacrifice by rinsing the lung with 600 µL of sterile saline via the endotracheal tube. BAL fluid was immediately centrifuged at 1,600xg at $4^0$C for 10 minutes. The supernatant was centrifuged again at 16,000xg at $4^0$C for 10 minutes to remove potential remaining cells and debris and stored at -80˚C until further analysis. DNA isolation and quantification of nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) by quantitative PCR was performed as described previously [8] using the following primer pairs (obtained from Biolegio, Nijmegen, the Netherlands): nDNA (GAPDH): Forward 5'-GCTATCTCATGTTCTTCAGAGTGG-3', Reverse 5'-TACCTTTGTTGTGGTACGTGCATA-3'. mtDNA: forward 5'-AATCGCACATGGCCTCACAT-'3 and reverse 5'-GAAGTCCTCGGGCCATGATT-'3. Samples were analyzed in duplicate and a fresh aliquot of DNA isolated from lung tissue homogenate of a non-ventilated mouse was used in each plate as a calibrator (between-plate CV% of 0.47% (GAPDH) and 0.36% (mtDNA)). BAL nDNA and mtDNA quantities are expressed as fold change relative to the expression of

the same gene in the calibrator sample.
For pulmonary cytokine determination, lung tissue from separate groups of wild-type and TLR9$^{-/-}$ mice was snap-frozen and stored at -80˚C, after which samples were homogenized in Tissue Protein Extraction Reagent (T-PER; Thermo Scientific, Rockford, USA) supplemented with complete EDTA-free protease inhibitor cocktail tablets (Roche Applied Science, Almere, The Netherlands) using the GentleMACS dissociater (Miltenyi Biotec, Leiden, The Netherlands). Homogenates were centrifuged (10 min, 16,000g, 4°C), and the supernatant was stored at −80°C until cytokine analysis. keratinocyte-derived chemokine (KC, murine equivalent of human interleukin (IL)-8) was determined using ELISA (R&D Systems, Minneapolis, MN, USA), IL-1β using a radioimmunoassay (RIA) as described previously [9], and IL-6, IL-10, and Tumor Necrosis Factor alpha (TNF-α) using a simultaneous Luminex assay (Milliplex, Millipore, Billerica, MA, USA). Cytokine concentrations were normalized to total protein content determined by bicinchoninic acid assay (BCA Protein Assay, Life Technologies, Bleiswijk, the Netherlands).

**Statistical analysis**
Data were not normally distributed, as determined by Shapiro-Wilk tests. Differences between groups were analyzed using Kruskal-Wallis and Dunn's post-hoc tests. Statistical analysis was performed using Graphpad Prism 5 software (Graphpad Software, La Jolla, USA). P-values <0.05 were considered significant.



## Results

Levels of nDNA, but not of mtDNA were increased after MV with tidal volumes of 8 or 32 mL/kg compared with unventilated control mice (Figure 1).
Furthermore, MV with both tidal volumes resulted in increased pulmonary concentrations of KC, IL-1β, and IL-6, all important cytokines in the MV-induced inflammatory response [10]. MV did not result in increased levels of IL-10 and TNF-α. Furthermore, no differences in pulmonary cytokine concentrations were observed between wild-type and TLR9$^{-/-}$ mice (Figure 2).

## Discussion

This study demonstrates that MV with either low or high tidal volumes does not result in mtDNA release into the pulmonary compartment. In accordance with this finding, TLR9 does not play a role in the MV-induced inflammatory response. Nevertheless, the finding of increased levels of nDNA, a marker for cell damage

and/or general DAMP release [8], supports previous findings that DAMPs may play a role in the MV-induced inflammatory response [3, 4]. The fact that we did not find increased mtDNA levels while these have been reported in patients with, and animal models of sepsis, trauma, and cardiac arrest could be explained by the different mechanisms of cell damage (mechanical stretch or disruption of the cell membrane by baro/volutrauma versus crush damage or ischemia of cells) [8, 11-15] Furthermore, the difference between MV-induced nDNA and mtDNA levels in our study suggests different mechanisms of release and/or clearance, which remain to be unraveled.

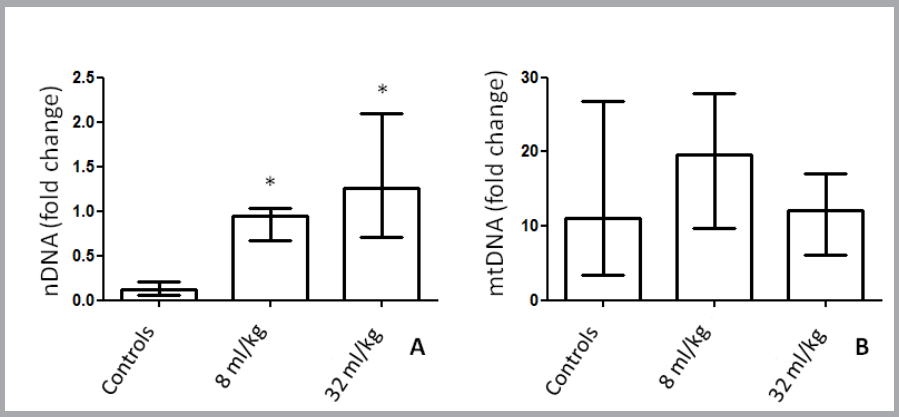


***Figure 1 – Nucleic acids in BAL fluid of wild-type mice*** *nDNA (A) and mtDNA (B) levels in BAL fluid. Data (n=8 per group) are expressed as median±IQR fold change (relative to a calibrator sample). * represents p<0.05 compared with the unventilated control group.*

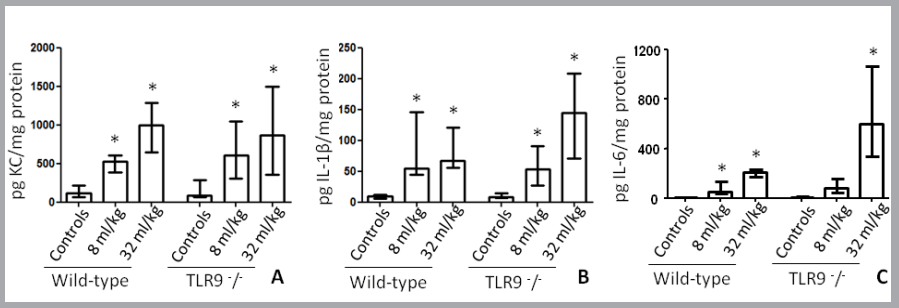


***Figure 2 – Cytokines in lung homogenates of wild-type and TLR9-deficient mice*** *KC (A), IL-1β (B), and IL-6 (C) concentrations in lung tissue homogenates. Data (n=8 per group) are expressed as median±IQR pg/mg of total protein. * represents p<0.05 compared with the corresponding unventilated control group.*

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# Chapter 8

## IL-1β processing in mechanical ventilation-induced inflammation is dependent on neutrophil factors rather than caspase-1

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## Abstract

**Purpose** Mechanical ventilation can cause ventilator-induced lung injury, characterized by a sterile inflammatory response in the lungs resulting in tissue damage and respiratory failure. The cytokine IL-1β is thought to play an important role in the pathogenesis of ventilator-induced lung injury. Cleavage of the inactive precursor pro-IL-1β to form bioactive IL-1β is mediated by several types of proteases, of which caspase-1, activated within the inflammasome, is most important. Herein, we studied the roles of IL-1β, caspase-1, and neutrophil factors in the mechanical ventilation-induced inflammatory response in mice.

**Methods** Untreated wild-type, IL-1αβ knockout and caspase-1 knockout mice, pralnacasan (a selective caspase-1 inhibitor)-treated mice, anti-KC-treated mice, and cyclophosphamide neutrophil-depleted wild-type mice were ventilated using clinically relevant ventilator settings (tidal volume 8 ml/kg). Lungs and plasma were collected to determine blood gas values, cytokine profiles and neutrophil influx.

**Results** Mechanical ventilation resulted in increased pulmonary concentrations of IL-1β and KC and increased pulmonary neutrophil influx compared with non-ventilated mice. Ventilated IL-1αβ knockout mice did not demonstrate this increase in cytokines. No significant differences were observed between wild-type and caspase-1 deficient or pralnacasan-treated mice. In contrast, in anti-KC antibody treated mice and neutropenic mice, inflammatory parameters were decreased in comparison with ventilated non-treated mice.

**Conclusions** Our results illustrate that IL-1 is indeed an important cytokine in the inflammatory cascade induced by mechanical ventilation. However, the inflammasome/caspase-1 appears not to be involved in IL-1β processing in this type of inflammatory response. The attenuated inflammatory response observed in ventilated anti-KC treated and neutropenic mice suggests that IL-1β processing in mechanical ventilation-induced inflammation is mainly mediated by neutrophil factors.

## Introduction

Mechanical ventilation is a life-saving therapy, although it can also cause ventilator-induced lung injury (VILI) [1]. VILI is characterized by a sterile inflammatory response in the lungs resulting in tissue damage that may sustain respiratory failure. The mechanical ventilation-induced inflammatory response can also spread systemically, which in severe cases can result in multi organ dysfunction syndrome (MODS) [2]. Even protective ventilation strategies that do not cause direct mechano-induced tissue damage (baro- or volutrauma) have been shown to elicit the release of pro-inflammatory cytokines, recruitment of leukocytes, and subsequent lung injury [3, 4]. The mechanisms behind this so-called 'biotrauma' have not yet been completely elucidated.

Previous studies have demonstrated that the TLR4/TRIF pathway is important in the mechanical ventilation-induced inflammatory response [4, 5]. Furthermore, it is becoming increasingly clear that the pro-inflammatory cytokine interleukin-1β (IL-1β) plays a key role in the pathogenesis of the inflammatory response and VILI by promoting neutrophil recruitment and by increasing epithelial injury and permeability [6-8]. Through recognition by the IL-1 receptor (IL-1R), secreted IL-1β, but also the cell-associated family member IL-1α, may stimulate production of other inflammatory cytokines via IL-1R-associated kinases (IRAKs) and thereby positively amplify the inflammatory response [9]. However, up till now this has not been studied in the context of mechanical ventilation-induced inflammation.

Upon activation of the innate immune system, e.g. via TLRs, IL-1β is synthesized as an inactive precursor molecule, pro-IL-1β, that cannot bind and activate the IL-1R [10]. In order to process pro-IL-1β and form bioactive IL-1β, proteolytic cleavage of the N-terminal 116 amino acids from the precursor is required. Caspase-1 is the major protein implicated in cleavage of pro-IL-1β [10, 11].

Caspase-1 exists as an inactive zymogen in cells of myeloid origin (e.g. tissue macrophages, dendritic cells) which needs to be activated to perform its proteolytic tasks [9]. Caspase-1 is also known to be expressed in a wide range of other cell types including lung fibroblasts and epithelial cells [12, 13]. The inflammasome is a protein platform that is responsible for the activation of caspase-1 [10, 14]. A broad range of infectious and autoimmune diseases that involve IL-1β have been associated with inappropriate activation of the inflammasome [12, 14, 15], while in several other disease models in which IL-1β plays a crucial role, the inflammasome appears not to be involved [16, 17]. IL-1β processing in these models might rely on neutrophil serine proteases, like elastase, granzyme A, cathepsin G or proteinase-3 [10, 18-20]. Hitherto, the role of caspase-1 in processing of IL-1β in the mechanical ventilation-induced

inflammatory response is unknown.
We studied the roles of IL-1β, caspase-1, and neutrophil factors in the mechanical ventilation-induced inflammatory response in mice ventilated with clinically relevant ventilator settings.

## Materials and methods

All experiments were approved by the Regional Animal Ethics Committee in Nijmegen and performed under the guidelines of the Dutch Council for Animal Care and the National Institutes of Health. They have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

### Animals

Age-matched wild-type C57Bl/6 mice and extensively backcrossed caspase-1 knockout mice or IL-1αβ knockout mice (aged 8-14 weeks, weight 25±4 grams) with C57Bl/6 background were used in this study. The mice were housed in a light and temperature controlled room under specific pathogen-free (SPF) conditions. Standard pelleted chow (1.00 % Ca; 0.22 % Mg; 0.24 % Na; 0.70 % P; 1.02 % K; SSNIFF Spezialdiäten GmbH, Soest, Germany) and drinking water were available *ad libitum*. These conditions are similar to previous studies in which this mouse model was used [4, 5, 21, 22].

### Experimental design

#### *IL-1αβ knockout experiments*

IL-1 can induce inflammation via activation of the IL-1 receptor. To study whether IL-1 is indeed involved in initiation and/or propagation of the inflammatory cascade induced by mechanical ventilation, mechanically ventilated IL-1αβ$^{-/-}$ mice (n=8) were compared with ventilated wild-type mice (n=8). As controls, non-ventilated IL-1αβ$^{-/-}$ (n=8) and wild-type mice (n=8) were used.

#### *Caspase-1 experiments*

Caspase-1 is able to cleave the inactive precursor pro-IL-1β to form the active cytokine IL-1β. To study the role of caspase-1 in the mechanical ventilation-induced inflammatory response, mechanically ventilated caspase-1 knockout mice (n=8) and ventilated wild-type mice treated with the selective caspase-1 inhibitor pralnacasan (100 mg/kg) (n=8) were compared with ventilated untreated wild-type mice (n=8) [23, 24]. As controls, non-ventilated caspase-1$^{-/-}$, pralnacasan-

treated wild-type and untreated wild-type mice (n=8 per group) were used.

***Anti-KC antibody experiments***

Apart from caspase-1, neutrophil serine proteases are also able to process IL-1β. In order to investigate whether the attraction of neutrophils by the chemo-attractant KC is involved in the inflammatory response elicited by mechanical ventilation, mechanically ventilated wild-type mice treated with an intraperitoneal dose of 100 µg of a neutralizing monoclonal anti-KC antibody (R&D systems, Minneapolis, MN, USA) 1 hour before induction of anaesthesia (n=8) were compared with ventilated untreated wild-type mice (n=8). As controls, non-ventilated untreated wild-type mice (n=8) were used.

***Neutrophil depletion experiments***

Neutrophil serine proteases are able to process IL-1β. In order to study the possible role of neutrophil factors in IL-1β processing in the mechanical ventilation-induced inflammatory response, mechanically ventilated neutrophil-depleted wild-type mice (n=8) were compared with ventilated untreated wild-type mice (n=8). As controls, non-ventilated wild-type mice (n=8) were used.

The neutrophil-depleted group was neutrophil-depleted with cyclophosphamide as described previously [25, 26].

**Experimental procedures**

Mice were anaesthetized using an intraperitoneal injection of 7.5 µl per gram body weight of KMA mix (25.5 mg/ml ketamine, 40 µg/ml medetomidine, 0.1 mg/ml atropine in saline). Subsequently, animals were orally intubated, an arterial line was placed in the arteria carotis, and the mice were mechanically ventilated (MiniVent®, Hugo Sachs Elektronik-Harvard apparatus, March-Hugstetten, Germany). The ventilation settings used were based on measured tidal volume and respiratory rate during spontaneous ventilation in C57Bl/6 mice [27]: a tidal volume of 8 ml/kg body weight and a frequency of 150/min. All animals received 4 cm H2O positive end-expiratory pressure (PEEP) and fraction of inspired oxygen was set to 0.4. In order to maintain anaesthesia, boluses of 5.0 µl per gram body weight maintenance KMA mix (3.6 mg/ml ketamine, 4 µg/ml medetomidine, 7.5 µg/ml atropine in saline) were given every 30 minutes via an intraperitoneally placed catheter. Rectal temperature was monitored continuously and maintained between 36.0$^{o}$C and 37.5$^{o}$C using a heating pad. After the 4 hour ventilation period, mice were sacrificed by exsanguination under anaesthesia. The control mice were anesthetized, but not ventilated and sacrificed shortly after induction

of anaesthesia. Tissue and blood were sampled in order to determine blood gas values (only in ventilated mice), cytokine production and neutrophil influx.
Lipopolysaccharide (LPS) was measured in the mechanical ventilation circuit by Limulus Amebocyte Lysate testing (Cambrex Bio Science, Walkersville, MD; detection limit: 0.06 IU/ml) to rule out contamination and LPS-induced pulmonary inflammation.

**Tissue harvesting**

Plasma was isolated by centrifugation at 13000xg for 5 min and stored at -80$^0$C. Immediately after exsanguination, heart and lungs were carefully removed en block via midline sternotomy. The right middle lung lobe was fixed in 4% buffered formalin solution overnight at room temperature. The right lung was snap frozen in liquid nitrogen and stored at -80$^0$C. The left lung was snap-frozen and placed in 500 µL lysis buffer containing PBS, 0.5% triton X-100 and protease inhibitor (complete EDTA-free tablets, Roche, Woerden, The Netherlands). Subsequently, the lungs were homogenized using a polytron and subjected to two rapid freeze-thaw cycles using liquid nitrogen. Finally, homogenates were centrifuged (10 minutes, 16000xg, 4 °C) and the supernatant was stored at -80° C until further analysis.

**Pulmonary neutrophil influx**

After overnight incubation in 4% buffered formalin solution, the right middle lung lobe was dehydrated, and embedded in paraplast (Amstelstad, Amsterdam, The Netherlands). Sections of 4 µm-thickness were used. Enzyme histochemistry using chloracetatesterase (LEDER staining) was used to visualize the enzyme activity in the neutrophils. Neutrophils were counted manually (10 fields per mouse), and after automated correction for air/tissue ratio, the average number of neutrophils/cm$^2$ per mouse was calculated.

**Biochemical analysis**

KC (keratinocyte-derived chemokine, murine equivalent of human IL-8) in lung homogenate was determined by enzyme-linked-immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA). Lower detection limit: 160 pg/ml. IL-1β in lung homogenate was determined using a radioimmunoassay (RIA) as described previously [28]. In samples of the IL-1αβ (Figure 1) and caspase (Figure 2) experiments, total protein concentrations in the lung homogenates were determined using a BCA protein assay (Thermo Fisher Scientific, Etten-Leur, The Netherlands) and cytokine concentrations in the homogenates were normalized

for protein concentration and therefore expressed as ng cytokine/µg protein. In anti-KC (Figure 3) and neutrophil depletion (Figure 4) experiments, cytokine concentrations in lung homogenate were not normalized for total protein content due to insufficient sample volume, and therefore expressed as pg/mL.

**Statistical analysis**

Data were not normally distributed (determined using Kolmogorov-Smirnov and Shapiro-Wilk tests) and therefore expressed as median and range or median and interquartile range [IQR]. Differences between groups were analyzed using Kruskal-Wallis and Dunn's post-hoc tests. Statistical analysis was performed using Graphpad Prism 5 software (Graphpad Software, La Jolla, USA). P-values $< 0.05$ were considered significant.

## Results

Mean arterial pressure remained stable and above 65 mmHg in all animals throughout the mechanical ventilation period. Blood gas values that were obtained at the end of the ventilation period did not indicate substantial lung injury (Table 1).

| | Median | IQR |
|---|---|---|
| **pH** | 7.36 | 7.25 – 7.38 |
| **PCO2** | 4.73 | 4.17 – 5.18 |
| **PO2** | 15.3 | 14.6 – 17.5 |
| **BE** | -5.5 | -7.3 – -4.0 |
| **HCO3** | 20.2 | 18.3 – 20.7 |
| **TCO2** | 21.0 | 19.8 – 21.5 |
| **sO2%** | 99% | 98 – 99 |
| **Lactate** | 0.98 | 0.90 – 1.16 |

***Table 1 – Blood gas values after 4 hours of ventilation*** *Values (median and interquartile range [IQR]) from a representative ventilated group (wildtype ventilated mice used as the control group for caspase-1$^{-/-}$ and pralnacasan-treated mice).*

**Involvement of IL1 in the mechanical ventilation-induced inflammatory response**

After 4 hours of mechanical ventilation, pulmonary levels of pro-inflammatory cytokine KC were significantly increased in wild-type mice compared with non-ventilated wild-type mice. In contrast, ventilated IL-1αβ knockout mice did not show an increase in pulmonary cytokines compared with non-ventilated IL-1αβ knockout mice (Figure 1).

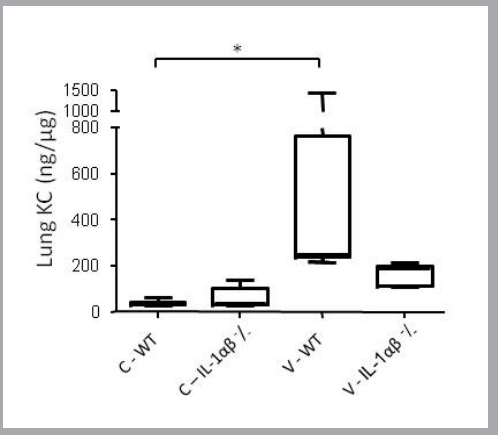


***Figure 1 – Involvement of IL1 in the mechanical ventilation-induced inflammatory response*** *KC levels in lung homogenates expressed as nanogram cytokine per microgram total protein. Data are expressed as box-and-whiskers plots, with min to max range as whiskers. Results of analysis in non-ventilated (C) and ventilated (V) wild-type (WT) mice and IL1αβ knock-out (-/-) mice are shown.*

**Involvement of caspase-1 in the mechanical ventilation-induced inflammatory response**

Pulmonary neutrophil influx was significantly increased in mechanically ventilated mice compared with non-ventilated wild-type and caspase-1$^{-/-}$ mice, but no differences were observed between wild-type mice, caspase-1$^{-/-}$ mice or pralnacasan-treated mice. Similar to the results described above, 4 hours of mechanical ventilation resulted in increased IL-1β and KC concentrations in lung homogenates in all groups. However, no significant differences in lung cytokine levels were observed between wild-type mice, caspase-1$^{-/-}$ mice or pralnacasan-treated mice. (Figure 2)

**Involvement of neutrophil factors in the mechanical ventilation-induced inflammatory response**

To determine whether neutrophil factors are involved in the mechanical ventilation-induced inflammatory response and IL-1β processing, we investigated the effects of treatment with an antibody against KC. KC is one of the major factors involved in neutrophil attraction to the site of inflammation (chemo-attractants). Mechanical ventilation resulted in increased levels of pulmonary neutrophils

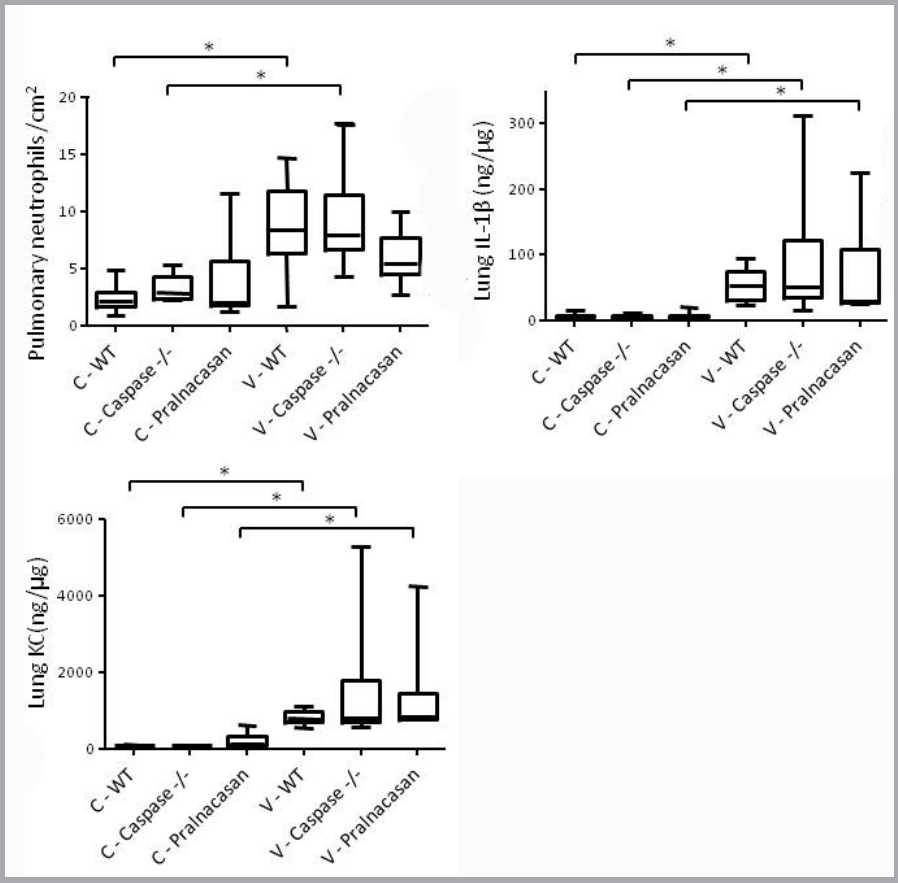


***Figure 2 – Involvement of caspase-1 in the mechanical ventilation-induced inflammatory response*** *Pulmonary neutrophil counts, expressed as number of neutrophils per cm2 tissue and IL-1β and KC levels in lung homogenates, expressed as nanogram cytokine per microgram total protein. Data are expressed as box-and-whiskers plots, with min to max range as whiskers. Results of analysis in non-ventilated (C) and ventilated (V) wild-type (WT) mice, caspase-1 knock-out (-/-) mice and pralnacasan-treated mice are shown.*

(Figure 3). This increase was abrogated by pre-treatment with an anti-KC antibody. Furthermore, the mechanical ventilation-induced increase in pulmonary IL-1β levels was less pronounced in anti-KC-treated mice compared with untreated mice, although this did not reach statistical significance (Figure 3).

To further confirm the role of neutrophil factors, we investigated the effects of mechanical ventilation following neutrophil depletion using cyclophosphamide. The effect of cyclophosphamide was visually inspected and no pulmonary neutrophils were present (data not shown). As depicted in Figure 4, the mechanical

ventilation-induced increase in pulmonary IL-1β and KC concentrations was diminished in neutrophil-depleted mice.

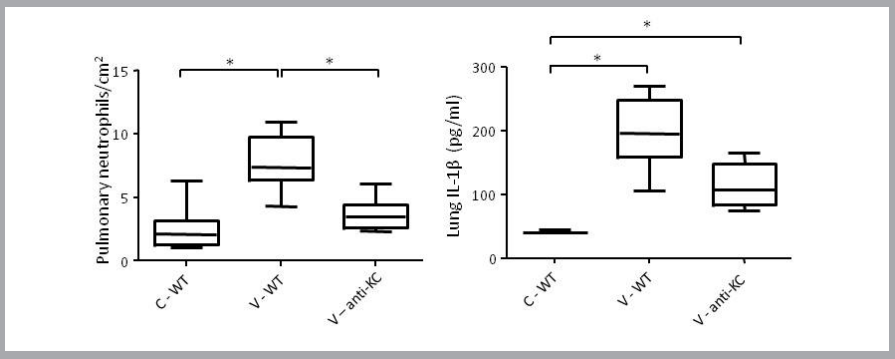


***Figure 3 – Involvement of KC in the mechanical ventilation-induced inflammatory response*** *Pulmonary neutrophil counts, expressed as number of neutrophils per cm2 tissue and IL-1β concentration expressed as pg cytokine/ml lung homogenate. Data are expressed as box-and-whiskers plots, with min to max range as whiskers. Pulmonary neutrophils and IL-1β concentration in non-ventilated (C) and ventilated (V) untreated wild-type mice (WT) and anti-KC antibody treated wild-type (anti-KC) mice are shown.*

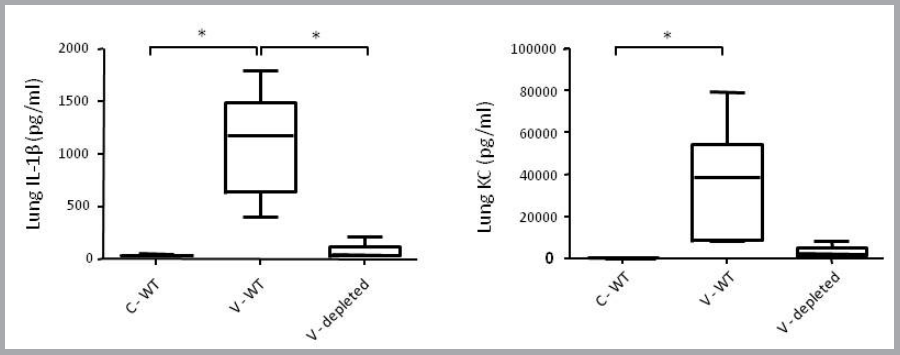


***Figure 4 – Effects of neutrophil depletion on the mechanical ventilation-induced inflammatory response*** *IL-1β and KC concentrations expressed as pg cytokine/ml lung homogenate, measured in non-ventilated (C) and ventilated (V) untreated wild-type (WT) and cyclophosphamide-treated neutrophil-depleted mice. Data are expressed as box-and-whiskers plots, with min to max range as whiskers.*

Our hypothesis regarding the role of IL-1β processing in the inflammatory response following mechanical ventilation is illustrated in Figure 5.

## Discussion

Consistent with previous results published by our group [4, 5, 22] and others [29, 30] the present study shows that mechanical ventilation using clinical relevant settings induces a pulmonary inflammatory response in mice. In addition, our data is in support of previous findings that IL-1 plays an important role in initiation and/or propagation of the mechanical ventilation-induced inflammatory response, and suggests that processing of IL-1β in mechanical ventilation-induced inflammation occurs via the release of neutrophil factors and not through caspase-1-dependent mechanisms.

Our finding that caspase-1 does not play a significant role in mechanical ventilation-induced inflammation is in contrast to a recent study where the NLRP3 inflammasome was found to play an important role in the mechanical ventilation-induced inflammatory response and VILI [31]. Several differences between their study and ours might explain the different results. First, in the previous study, ASC and NLRP3 (components of the inflammasome upstream of caspase-1) knockout mice were used and it was shown that mechanical ventilation activated caspase-1 in a NLRP3-dependent fashion. Nevertheless, it is very well possible that ASC and NLRP3 play other roles in the mechanical ventilation-induced inflammatory cascade than merely activating caspase-1. As abrogation and inhibition of caspase-1 by either a knockout approach or pralnacasan treatment did not have any effect in our model, the role of caspase-1/the inflammasome appears not to be as crucial as suggested. Second, differences between wildtype and ASC or NLRP3 knockout were only found at a high tidal volume of 15 ml/kg, known to cause extensive lung damage [22], while no effects were found at a low tidal volume of 7.5 ml/kg, which is more representative of the current clinical practice and similar to that used in the present study. This suggests that the inflammasome might play a more important role at higher tidal volumes which lead to apparent lung injury but not in mechanical ventilation-induced inflammation at clinically relevant ventilator settings. Interestingly, a more recent study from the same group showed that pre-treatment with allopurinol or uricase (both degraders of known inflammasome-activating factors [32] did not decrease mechanical ventilation-induced inflammation, which is in support of a caspase/inflammasome-independent mechanism [33]. As beneficial effects of uricase and allopurinol were observed in terms of alveolar barrier dysfunction, it appears plausible that ASC and NLRP3 are involved in VILI via inflammation-independent mechanisms.

The pronounced influx of neutrophils in the lung observed in our experiments

suggests a major role for these inflammatory cells in the inflammatory cascade following mechanical ventilation. Our findings that treatment with an antibody against KC or depletion of neutrophils reduced the mechanical ventilation-induced production of IL-1β and KC indicate an important role for neutrophils in initiation and/or propagation of the inflammatory response. In this respect, pro-IL-1β cleavage in our model is probably achieved through neutrophil factors, such

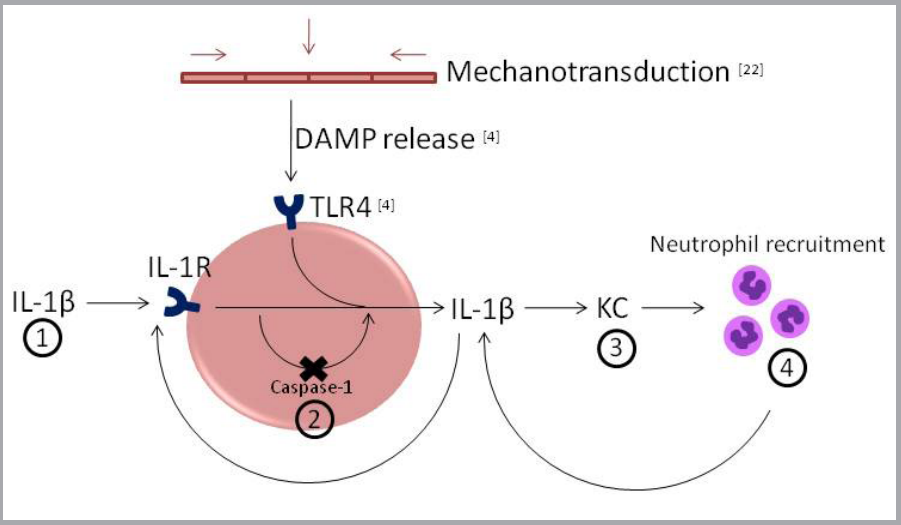


***Figure 5 – Hypothesis regarding the role of IL-1β processing in the inflammatory response following mechanical ventilation.*** *We present the following hypothesis based on our results and previous findings. Mechanical ventilation causes mechanotransduction and cell and/or tissue damage. This causes the release of DAMPs (Danger Associated Molecular Patterns), that activate TLR4 and possibly other pattern recognition receptors. Ligation of these receptors induces production of cytokines, most importantly IL-1β. Subsequently, KC is produced, leading to neutrophil recruitment to the lungs. Pro-IL-1β processing to bioactive IL-1β could occur intracellularly by caspase-1, although in our model, it only plays a minor role in IL-1β bioactivation, not excluding that it may be involved at the onset of the inflammatory process, when very few neutrophils are present. The majority of pro-IL-1β is excreted in the inactive form and then cleaved by factors released by neutrophils, such as neutrophil serine proteases. Finally, active IL-1β present extracellularly binds to the IL-1R, which in turn leads to the production of more cytokines and hence, positive amplification of the inflammatory response. As such, a positive feedback loop is activated which could be an explanation for the extensive inflammatory response observed following mechanical ventilation.*
*Numbers 1 to 4 represent the experiments performed in this study and correspond to the figure numbers in this paper. References 4 and 22 refer to previous studies performed by our group.*

as the serine proteases proteinase-3 (PR-3), elastase or cathepsin G, leading to bioactive IL-1β and propagation of the inflammatory response through binding of the IL-1-receptor, which in turn leads to production of other inflammatory cytokines such as KC [10, 34, 35]. Several other IL-1β mediated inflammatory responses are described to be partly or completely independent of the inflammasome and caspase-1, and possibly dependent on neutrophil factors, including proteinase 3 and cathepsin G [35]. Future studies should focus on the confirmation of our hypothesis and the identification of these neutrophil factors.

Our study has several limitations. First, we used cyclophosphamide to deplete neutrophils. While this is a widely used method[25, 26, 36, 37], cyclophosphamide treatment may also result in depletion of other cell types that play a role in mechanical ventilation-induced inflammation [38, 39]. Nevertheless, our data of mice treated with an anti-KC antibody underline the importance of neutrophils in this process. Second, no histological slides to perform neutrophil counts were collected in the IL-1αβ$^{-/-}$ experiments to investigate whether these knockout mice were still able to recruit neutrophils. Finally, we can not exclude the possibility that, next to mechanical ventilation, the procedures related to the instrumentation/ ventilation (e.g. intubation, arterial cannulation) also induce inflammation to a certain extent. However, we have previously shown that the inflammatory response is aggravated when mice are ventilated with these parameters for a longer period of time or when higher tidal volumes are used, suggesting that the inflammatory response is mainly ventilation-induced.

In conclusion, our results indicate that IL-1 signaling is important in mechanical ventilation-induced inflammation. We show that following mechanical ventilation, IL-1β bioactivation is not caspase-1 dependent, but appears to be mediated by neutrophil factors, leading to a positive amplification loop and further propagation of the inflammatory response. Further elucidation of the precise mechanism of IL-1β processing in mechanical ventilation-induced inflammation could provide novel targets for the future treatment of VILI [40].

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# Part III

## Biomarkers in trauma

# Chapter 9

## Circulating iFABP levels as a marker of intestinal damage in trauma patients

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## Abstract

**Background** Both the initial trauma and the subsequent hemodynamic instability may contribute to intestinal damage, which is of great importance in (immunological) post-trauma complications. The study assesses intestinal damage using the biomarker intestinal Fatty Acid Binding Protein (iFABP) in trauma patients during the first days of their hospital admission and the risk factors involved.

**Methods** Plasma iFABP levels were measured in blood samples obtained from adult multiple trauma patients (n=93) at the trauma scene by the helicopter emergency medical services (HEMS), at arrival at the emergency room (ER), and at days 1, 3, 5, 7, 10, and 14 after trauma and related to injury severity and hemodynamic parameters.

**Results** Plasma iFABP concentrations showed highest levels immediately following trauma at time points HEMS and ER. Non-survivors demonstrated higher iFABP levels at the ER compared with survivors. Furthermore, iFABP values at the ER correlated with ISS and patients suffering from abdominal trauma demonstrated significantly higher iFABP concentrations in comparison to patients with other types of trauma or healthy controls. Also, patients presenting with a MAP <70 mmHg at the ER, demonstrated significantly higher plasma iFABP concentrations in comparison with patients with a normal (70-99 mmHg) or high (>100 mmHg) MAP, or healthy controls. Finally, patients with a low Hb (<80% of reference value) displayed significantly higher iFABP concentrations in comparison with patients with a normal Hb or healthy controls.

**Conclusions** Plasma iFABP levels, indicative of intestinal injury, are increased immediately after trauma in patients with abdominal trauma, low MAP, or low Hb, and are related to the severity of the trauma. As intestinal injury is suggested to be related to late complications, such as MODS or sepsis in trauma patients, strategies to prevent intestinal damage following trauma could be of benefit to these patients.

## Introduction

Trauma is one of the main causes of death worldwide. In 2000, 9% of deaths and 12% of disease burden could be attributed to trauma. Moreover, 50% of trauma-related deaths are young people between the ages of 15 and 44 years [1, 2]. Roughly, trauma deaths can be divided into two types. Early deaths can be attributed to the direct effect of trauma, such as blood loss or neurological damage. Late mortality on the other hand, is to an important extent due to complications secondary to the trauma [3, 4].

Among other organ systems, the digestive system is affected by the trauma. Direct abdominal trauma, ischemia induced by hemodynamic insufficiency, or a systemic inflammatory response can result in intestinal damage. Mesenteric ischemia has high mortality numbers and is very difficult to diagnose in an early stage [5]. One of the deleterious consequences of intestinal damage is altered gut permeability, which can result in translocation of bacteria [6, 7]. The transit of bacteria through the intestinal wall can subsequently lead to a hyperinflammatory response, and ultimately, multi organ dysfunction syndrome (MODS) [7, 8].

Intestinal Fatty Acid Binding Protein (IFABP) is a protein exclusively expressed by enterocytes. It is released into the circulation following enterocyte damage and has shown to be a good marker for the early identification of intestinal ischemia and damage [9, 10].

Although previous studies have demonstrated increased intestinal permeability to be related to trauma severity [9, 11, 12], the timeframe in which this intestinal damage occurs remains unknown. Moreover, it has been described that the presence of shock could induce intestinal damage. However, it remains unclear whether this effect is attributable to low hemoglobin levels due to hemorrhage or low blood pressure, or both. The aim of this study was to assess the temporal relationship between trauma and intestinal damage using the biomarker iFABP during the first days of hospital admission, and to determine which factors play a role in the development of intestinal damage in these patients.



## Methods

The study was carried out in the Netherlands in accordance with the applicable rules concerning the review of research ethics committees and informed consent. The protocol was approved by the local ethics committee of the Radboud University Medical Centre (protocol ID NL38169.091.11). All patients or their legal representatives were informed about the study details. Written informed consent

was obtained in the event that venipuncture was necessary to obtain blood samples.
All study procedures were performed under the guidelines of The National Institutes of Health and in accordance with the declaration of Helsinki and its later amendments.

**Subjects**
Adult trauma patients (n=93) admitted to the trauma care unit at the emergency room (ER) of the Radboud University Medical Centre between march 2011 and may 2012 were included in the study. Exclusion criteria were known HIV/AIDS, malignancies, and use of steroids or other immunosuppressive medication.
Follow-up was prematurely terminated mainly because of death, discharge, transfer to another hospital or because no written informed consent could be obtained.
Samples from 13 healthy volunteers (age median 27 [range 22 - 34], 4 female, 9 male) were used as controls.

**Sample collection**
Blood was sampled shortly after trauma by the Helicopter Emergency Medical Services (HEMS) (if present on the trauma scene and deemed feasible by the trauma physician on site), at arrival at the Emergency Room (ER), and at days 1, 3, 5, 7, 10, and 14 following trauma. Lithium Heparin anticoagulated blood was centrifuged after withdrawal at 1,600 x g at 4$^{0}$C for 10 minutes, after which plasma was stored at -80$^{0}$C until further analysis.

**Data collection**
Plasma iFABP concentrations were determined by ELISA (detection limits 187.2-12000 pg/ml) according to the manufacturers' instructions (HyCult Biotech, Uden, the Netherlands).
Injury Severity Scores (ISS) were obtained via the regional trauma administration. Patient characteristics, injury mechanisms, Simplified Acute Physiology Score II (SAPS II) scores, hemoglobin (Hb) levels and mean arterial pressure (MAP) were obtained from the electronic patient file.

**Data analysis**
Distribution of data was determined using Kolmogorov-Smirnov tests. In order to calculate the deviation from normal Hb, patient Hb values were divided by the reference values (males 6.8 mmol/L, females 6.0 mmol/L), yielding a percentage

of reference Hb level per patient.
Groups were compared using Mann-Whitney U tests or Kruskal-Wallis tests with Dunn's multiple comparison post hoc test, as appropriate. After log transformation of iFABP data, Pearson correlation coefficients were calculated between ISS and plasma iFABP levels. Statistical analysis was performed using Graphpad Prism 5 software (Graphpad Software, La Jolla, USA). A p value of <0.05 was considered statistically significant.

## Results

### Patient characteristics

A total of 93 patients were included, of which the characteristics are listed in Table 1. The majority of patients suffered from head/neck injury and/or chest injury, and fall from height was the predominant injury mechanism. A total of 16/93 patients (17%) suffered abdominal trauma. Two patients of the study population developed sepsis at the ICU.

### iFABP levels after trauma

Plasma iFABP concentrations showed highest levels at time points HEMS and ER. Concentrations decreased in the following days, with a significant decrease observed at day 3 compared with time points HEMS and ER. (Figure 1A) No statistically significant difference was observed between healthy controls and all trauma patients combined, although in subgroups of trauma patients significantly higher iFABP levels were observed (detailed below).
Non-survivors demonstrated significantly higher iFABP levels at ER in comparison with survivors (Figure 1B). No differences in ER iFABP levels were observed between males and females (p=0.9). Furthermore, no correlation was observed between age and ER iFABP levels (r=-0.01, p=0.9).



### Relation between the severity and nature of trauma and iFABP levels

Injury Severity Score (ISS) was positively correlated with the plasma iFABP concentration measured at the ER (Figure 1C, r=0.34, p=0.002), indicating that more severe trauma is associated with higher iFABP levels. Furthermore, patients suffering from abdominal trauma displayed significantly higher plasma iFABP concentrations at the ER in comparison with patients with non-abdominal trauma at the same time point (Figure 2A). Moreover, patients with abdominal trauma demonstrated significantly higher iFABP levels in comparison with healthy controls at both time points HEMS and ER (p=0.04, n=16 and p=0.0004, n=14 respectively).

| | |
|---|---|
| Gender | Male: N=64 (68.8%)<br>Female: N=29 (31.2%) |
| Age (median [range]) | 50 [18-95] |
| 28-day survival | N=71 (76.3%) |
| ICU length of stay (median [range]) | 3 [0-35] days |
| Injury Severity Score (median [range]) | 25 [1-66] |
| Head/Neck injury (ISS region 1) | N=71, 76.3% |
| Face injury (ISS region 2) | N=25, 26.9% |
| Chest injury (ISS region 3) | N=54, 58.1% |
| Abdomen or pelvic contents injury (ISS region 4) | N=27, 29% |
| Abdominal trauma (AIS region 5) | N=16, 17.2% |
| | Score 2: N=7 |
| | Score 3: N=7 |
| | Score 4: N=2 |
| Extremities or pelvic girdle injury (ISS region 5) | N=43, 46.2% |
| External injury (ISS region 6) | N=45, 48.4% |
| Trauma Mechanism | |
| Fall from height | N=27, 29.0% |
| Car/Truck accident | N=25, 26.9% |
| Pedestrian/Cyclist accident | N=24, 25.8% |
| Motorcycle accident | N=10, 10.8% |
| Penetrating injury | N=5, 5.4% |
| Burn | N=1, 1.1% |
| Industrial accident | N=1, 1.1% |

***Table 1 – patient characteristics***

When classifying patients with abdominal trauma using the abbreviated injury scale (AIS) [13], more severe abdominal trauma appeared to be associated with higher plasma iFABP concentrations at the ER, however, statistical analysis was not possible due to the low number of patients with abdominal trauma (Figure 2B).

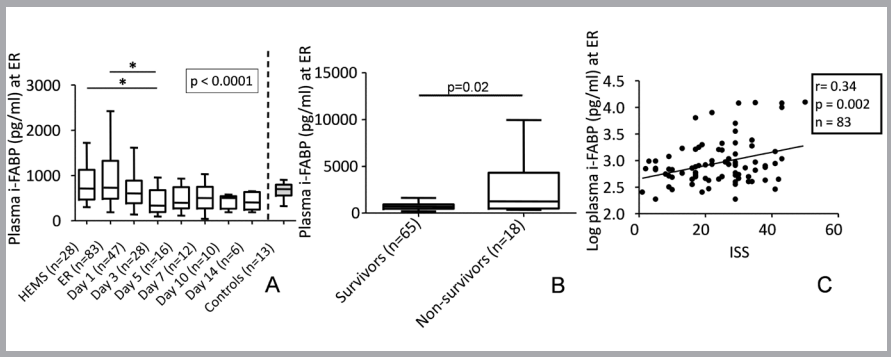


***Figure 1 - iFABP levels after trauma*** *A. Plasma iFABP concentrations in trauma patients and healthy controls. Data are represented as median and interquartile range. p-value calculated using Kruskall-Wallis test and * indicates p<0.05 using Dunn's post-hoc test. HEMS = Helicopter Emergency Medical Services, ER = Emergency room. B. Plasma iFABP concentrations in trauma patients at the ER in survivors and non-survivors. P value calculated using Mann-Whitney test. C. Correlation between Injury Severity Score (ISS) and plasma iFABP levels at the ER. R and p-values calculated using Pearson correlation.*

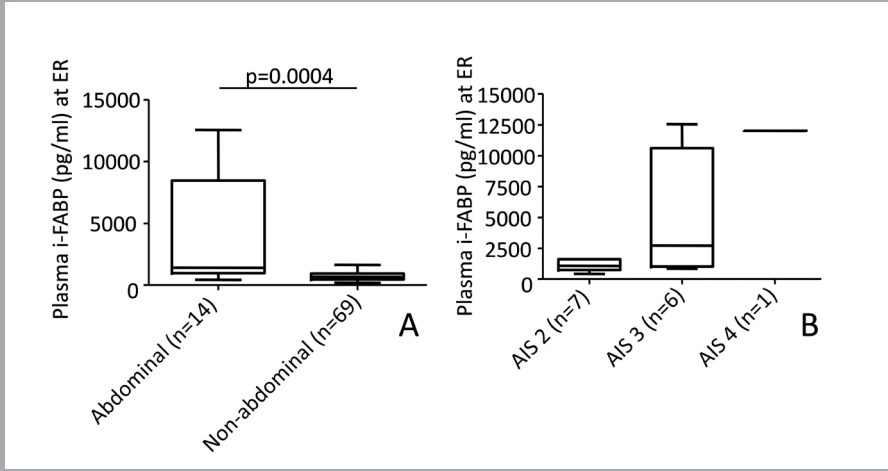


***Figure 2 - Relation between the nature and severity of trauma and iFABP levels*** *A. Plasma iFABP concentrations measured at the ER in patients with abdominal trauma and non-abdominal trauma. Data are represented as median and interquartile range. p-value calculated using Mann-Whitney U-test. B. Plasma iFABP concentrations measured at the ER in relation to abdominal abbreviated injury scale (AIS).*

**Relation between hemodynamic parameters and iFABP levels**

Patients with a MAP below 70 mmHg at the ER displayed higher iFABP levels compared with patients with a normal (70-100 mmHg) or high (>100 mmHg) MAP at the ER (Figure 3A). Patients with a MAP below 70 mmHg showed higher iFABP levels compared with healthy controls at both time points HEMS and ER (p=0.02, n=3 and p=0.007, n=8, respectively). Furthermore, patients with an Hb lower than 80% of the lower limit of the normal value for their sex showed increased iFABP levels compared with patients with an Hb level that was higher than 80% of the normal value (Figure 3B). Patients with an Hb < 80% of reference value displayed significantly higher iFABP levels compared with healthy controls at both time points HEMS and ER (p=0.04, n=5 and p=0.0004, n=13, respectively). No correlation was observed between Hb at ER and MAP at ER (r=0.09, p=0.34).

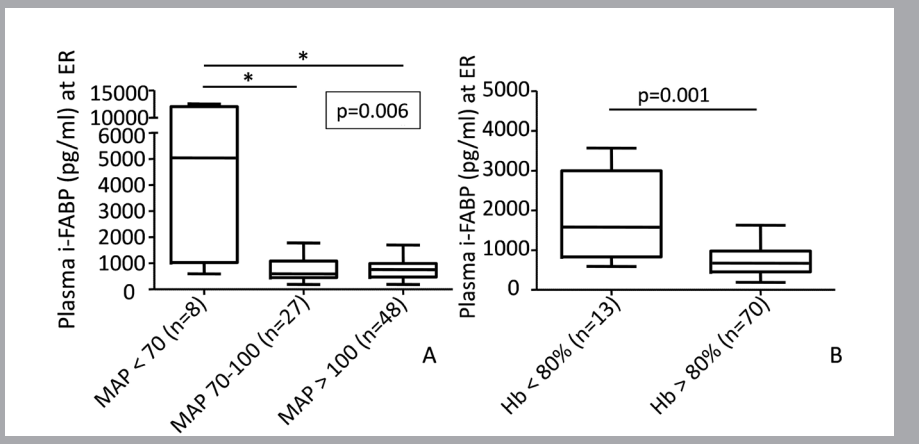


***Figure 3 – Relation between hemodynamic parameters and iFABP levels*** *A.Plasma iFABP concentrations measured at the ER in patients with a MAP lower than 70 , between 70 and 100 mmHg, and higher than 100 mmHg. Data are represented as median and interquartile range. p-value calculated using Kruskall-Wallis test and * indicates p<0.05 using Dunn's post-hoc test. B. Plasma iFABP concentrations measured at the ER in patients with Hb below and above than 80% of the normal values. Normal values for males and females are 6.8 mmol/L and 6.0 mmol/L, respectively. Data are represented as median and interquartile range. p-value calculated using Mann-Whitney U-test.*

## Discussion

This study demonstrates that major trauma results in very early release of iFABP in the circulation. To our knowledge, we present the first evidence that compared with healthy controls, iFABP levels are already profoundly elevated at the trauma scene before hospital admission in patients with abdominal trauma and signs of hemorrhage (low blood pressure or Hb), and that iFABP levels are higher in non-survivors compared with survivors. Furthermore, the amount of iFABP released is related to injury severity, presence of abdominal trauma, and signs of hemorrhage. The latter findings confirm previous research indicating elevated iFABP levels in trauma patients are related to injury severity and shock [9, 12].

Our results concerning the relation between increased iFABP levels, indicative of intestinal injury, and mortality are in line with a previous study in a shock and surgical trauma two-hit model in baboons showing a relation between increased gut permeability assessed by plasma D-lactate measurements and mortality in a shock and surgical trauma two-hit model [14]. In addition, this similarity further strengthens the suggestion that iFABP is a marker of gut permeability [9, 10].

Intestinal integrity is thought to play a major role in post-trauma complications [2]. Therefore, therapeutic interventions aimed to limit intestinal damage could be of benefit to trauma patients. We show substantial differences in plasma iFABP levels between patients with abdominal trauma and low Hb/MAP and patients with other trauma types and normal/high Hb/MAP. Previous studies in both trauma patients and a hemorrhagic shock model in rats display similar results, relating blood pressure with increased gut permeability or damage [12, 15]. Therefore, targeted interventions, such as (more aggressive or more goal-directed) fluid resuscitation and/or hemodynamic support, may represent a viable treatment option in these subgroups of patients. Currently, iFABP is not used as a biomarker in clinical practice, but these observational correlations between shock and iFABP concentrations and the known relationship between intestinal damage and the increased incidence of late complications in trauma patients indicate that treatment focused on intestinal perfusion might be worthwhile. For instance, a previous study demonstrated that circulatory management based on goal-directed fluid administration to maintain the MAP above 60 mmHg during non-abdominal surgery in children results in adequate intestinal perfusion and normal plasma iFABP levels without increasing the volume of fluids administrate [16]. Currently, no studies are available that demonstrate that a therapeutic strategy

can to influence circulating iFABP levels and improve clinical outcome in trauma patients or any other patient group. Timing of intervention could be of interest for future studies, as our results display increase iFABP levels already in a pre-hospital stage. Previous studies have suggested that fluid therapy before surgical intervention can control bleeding and could be able to improve outcome [17]. Moreover, as Hb levels appear to be related to iFABP levels independently of MAP, pre-hospital blood transfusion rather than crystalloids or colloids in selected patients might be considered [18-20]. Ongoing research addresses the question of what fluid treatment is most beneficial in hemorrhagic shock patients.

The present study has several limitations we want to acknowledge. Inherent to this type of study, a substantial number of patients were lost to follow-up, because of discharge from the hospital, transfer to another hospital, or death. Another weakness of the current study is the heterogeneity of the patients, which is typical for the multi-trauma patient population studied. Finally, the incidence of MODS and sepsis in our patient cohort was too low to relate these events to iFABP levels.

From this study we conclude that high ISS, abdominal trauma, and low Hb or MAP are associated with increased iFABP levels and thus intestinal injury that is present very early after trauma as well as at presentation on the ER. As intestinal injury is suggested to be related to late complications, such as MODS or sepsis in trauma patients, strategies to prevent intestinal damage following trauma could be of benefit to these patients.

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# Chapter 10

## SuPAR levels are related to plasma cytokine levels but have low predictive value for mortality in trauma patients

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## Abstract

**Introduction** Soluble urokinase-type plasminogen activator (suPAR) represents a marker for immune activation and has predictive value in critically ill patients. The kinetics of suPAR and its correlation with the immune response and outcome in trauma patients are unknown.

**Methods** Plasma concentrations of inflammatory cytokines and suPAR were determined in adult trauma patients (n=69) samples obtained by the Helicopter Emergency Medical Services (HEMS), at arrival at the Emergency Room (ER), and at day 1, 3, 5, 7, 10 and 14.

**Results** Initial SuPAR levels were unrelated to injury severity score and higher in non-survivors compared with survivors, although no difference was observed between early and late mortality. The area under the ROC curve to predict mortality was 0.6 (95%CI 0.48–0.72). SuPAR levels increased over time in 94% of patients, although suPAR increase did not precede death. TNF-α at the ER correlated with suPAR at that time point, while concentrations of other pro-inflammatory cytokines at the ER correlated with suPAR levels at days 1 and 5.

**Conclusions** After trauma, initial suPAR plasma concentrations are higher in non-survivors compared with survivors, but its predictive value is low. SuPAR levels increase over time after trauma, and concentrations at later timepoints are related to cytokine levels at the ER.

## Introduction

Trauma is one of the main causes of death worldwide. In 2000, 9% of deaths and 12% of disease burden could be attributed to trauma. Moreover, 50% of trauma-related deaths concern young people between the ages of 15 and 44 years [1]. Roughly, trauma deaths can be divided into early deaths attributable to the direct effect of trauma, such as blood loss or neurological damage and late mortality, to an important extent due to immunological complications [2, 3]. Trauma can result in a systemic inflammatory response, predominantly induced by the release of danger associated molecular patterns (DAMPs) [4], These "danger signals" can either be components from ruptured cells and tissues or factors released by cells in stressed conditions [4]. Pattern Recognition Receptors (PRRs), which recognize pathogen associated molecular patterns (PAMPs), can be triggered by DAMPs. Although the exact mechanisms are not completely elucidated yet, this can lead to pro-inflammatory responses as well as a refractory state of the immune system called "immunoparalysis", which renders patients vulnerable towards secondary infections [5]. Apart from injury severity scores, no predictors of mortality are available for this important patient group.

Soluble urokinase-type plasminogen activator receptor (suPAR) is a recently identified biomarker for inflammation in infectious diseases that is superior in predicting outcome in various conditions compared with other markers [6, 7]. SuPAR particularly appears to be of value in combination with other biomarkers [6, 8]. The non-soluble form of suPAR, urokinase plasminogen activator receptor (uPAR or CD87) is expressed on immune cells, e.g. neutrophils, monocytes, macrophages and lymphocytes [9] and does play a role in fybrinolytic pathway [10, 11]. Plasma levels of suPAR are increased in various infectious and inflammatory diseases [12-14]. Moreover, several observational studies demonstrate the relation between increased suPAR levels and outcome in critically ill patients [8, 15, 16]. However, suPAR levels in trauma patients and their relation with outcome have not been investigated.

Considering the importance of the immune response in late mortality of trauma patients, and the high prognostic value of suPAR in inflammatory conditions, the objective of this study was to investigate the prognostic value (for both early and late mortality) of plasma suPAR levels in trauma patients and to study the relation with markers of systemic inflammation and injury severity in this specific group of patients.

## Materials and Methods

### Study population

Adult trauma patients (n=69) admitted to the trauma care unit at the ER of the Radboud University Nijmegen Medical Center, a level I trauma center, were included in the study. Exclusion criteria were expected clinical risks of blood sampling, known HIV/AIDS, known malignancies and use of steroids or other immunomodulating medication. Two healthy male volunteers (aged 20 and 25 yrs) were used as healthy controls.

The study has been carried out in the Netherlands in accordance with the applicable rules concerning the review of research ethics committees and informed consent. All patients or legal representatives were informed about the study details at the first opportunity, usually at day 1 after admission. The local ethical committee that approved the study protocol agreed that it was not possible to do this at an earlier stage. Written informed consent was obtained if venapuncture was necessary to obtain blood samples. Informed consent was waived in case blood could be sampled from existing arterial or venous catheters. All experiments were performed under the guidelines of The National Institutes of Health and in accordance with the declaration of Helsinki and its later amendments.

### Sample collection

Blood was sampled shortly after trauma by the Helicopter Emergency Medical Services (HEMS), at arrival at the Emergency Room (ER), and at day 1, 3, 5, 7, 10 and 14 following trauma. Ethylenediaminetetraacetic acid (EDTA) anticoagulated blood was centrifuged after withdrawal at 1,600 x g at $4^{0}$C for 10 minutes, after which plasma was stored at -$80^{0}$C until further analysis.

### Sample analysis

Plasma concentrations of suPAR were analyzed in one batch by ELISA (detection limits 1.1 – 22.5 ng/ml) according to the manufacturer's instructions (SuPARnostic, Virogate A/S, Birkerød, Denmark). Plasma concentrations of Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-6, IL-10, IL-8, Interferon (IFN)-γ, and Monocyte Chemoattractant Protein (MCP)-1 were analyzed by a Luminex assay according to the manufacturer's instructions (Milliplex; Millipore, Billerica, MA, USA). Clinical parameters and demographic data were obtained from electronic patient files. Injury Severity Scores (ISS) and Abbreviated Injury Scale (AIS) were supplied by the Regional Emergency Healthcare Network.

### Statistical analysis

Data are expressed as mean±sem or median [IQR], according to their distribution as determined using the Kolmogorov-Smirnov test. Statistical tests used are indicated in the figure/table legends or text. All analyses were performed with available data of the corresponding time-points. Due to missing values at certain time points or patients that were lost to follow-up, patient numbers in the various analyses might not correspond with the original inclusion number.

Linear regression coefficients were calculated from patients that had a follow up of 3 or more days (n=33) to quantify suPAR kinetics over time.

A Receiver Operating Characteristics curve according to DeLong [et al], sensitivity, specificity, positive predictive value and negative predictive value were calculated using MedCalc version 11.3.1.0 (MedCalc software, Ostend, Belgium).

Other statistical analyses were performed using Graphpad Prism 5 software (Graphpad Software, La Jolla, USA). A p value of $<0.05$ was considered statistically significant.

## Results

### Patient characteristics

A total of 69 patients were included, of which the majority suffered from head/neck injury and/or chest injury. 3 out of 69 patients suffered from penetrating injury. Patient characteristics shown in Table 1.

### Plasma suPAR concentrations after trauma

Mean plasma suPAR level at time point HEMS was 3.2±0.4 ng/ml and significantly increased over time (Figure 1A), further illustrated by positive regression coefficients in 94% of patients with a minimum follow up of 3 days (mean regression coefficient of 0.4±0.1 ng/mL/day, Figure 1B). Furthermore, mean plasma suPAR levels in patients were significantly different from mean suPAR levels found in healthy controls (2.7 ng/ml) from time point ER onwards (Figure 1A). The concentrations we found in samples from healthy controls were similar to those found in healthy controls in previous studies [17-19].

There was no correlation (r=0.12, p=0.38) between suPAR and injury severity score (ISS). A weak, but statistically significant correlation was found between age and suPAR (r=0.29, p=0.02).

| | |
|---|---|
| Gender | Male: n=48 (69.6%)<br>Female: n=21 (30.4%) |
| Age (median [range]) | 50 [18-95] |
| Length of Stay in days (median [range]) | 9 [0-201] |
| 28-day survival | N=50 (72.5%) |
| Injury Severity Score (median [range]) | 29 [3-66] |
| Head/Neck injury (ISS region 1) | N=53, 80.3% |
| Face injury (ISS region 2) | N=17, 25.8% |
| Chest injury (ISS region 3) | N=42, 63.3% |
| Abdomen or pelvic contents injury (ISS region 4) | N=24, 36.4% |
| Extremities or pelvic girdle injury (ISS region 5) | N=40, 60.6% |
| External injury (ISS region 6) | N=31, 47.0% |

***Table 1 – patient characteristics***

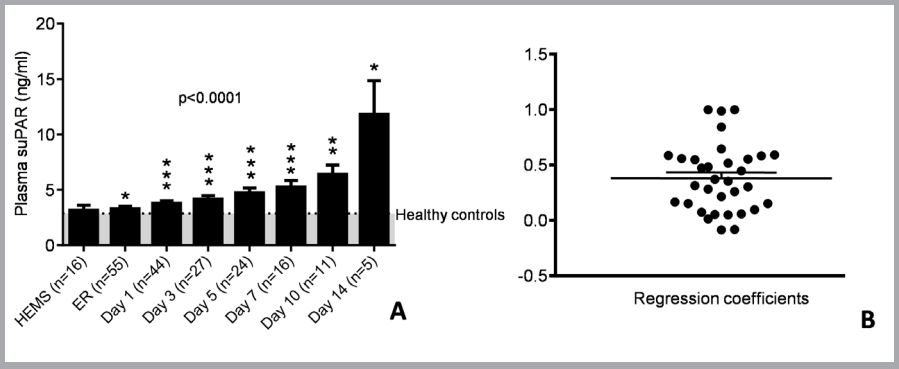


***Figure 1 – suPAR levels after trauma*** *A. Plasma suPAR levels were measured at 8 different time points following trauma. SuPAR levels increased over time (p<0.0001) according to one way ANOVA and were significantly higher than values of healthy controls (indicated in grey/ dotted-line) according to Bonferroni's multiple comparison post-hoc test. Data are expressed as mean±SEM. Median age per time point is displayed in years (yrs). * p<0.05, ** p<0.01, *** p<0.001 compared with healthy controls B. Regression coefficients were generally positive (n=33, mean±SEM 0.38±0.05), indicating increasing suPAR concentrations over time.*

**Relation beween suPAR levels and early and late mortality**

Initial suPAR levels (first sample of a patient, obtained at either time point HEMS, ER or day 1) were higher in non-survivors (n=22, 4.1±0.5 ng/ml) compared with survivors (n=45, 3.0±0.2 ng/ml, p=0.02). Furthermore, patients that died early (within 5 days) as well as those that died later (after 6 or more days) demonstrated

higher suPAR levels in comparison with survivors (Figure 2A), although this difference was only significant between the survival and early mortality groups. No statistically significant difference in suPAR levels between the early and late mortality groups was observed.

There was no difference in the increase in suPAR concentration between survivors and non-survivors, as regression coefficients between were similar (Figure 2B). Finally, no differences were observed in suPAR levels shortly before patients died, indicating that death itself is not preceded by an increase in suPAR (Figure 2C).

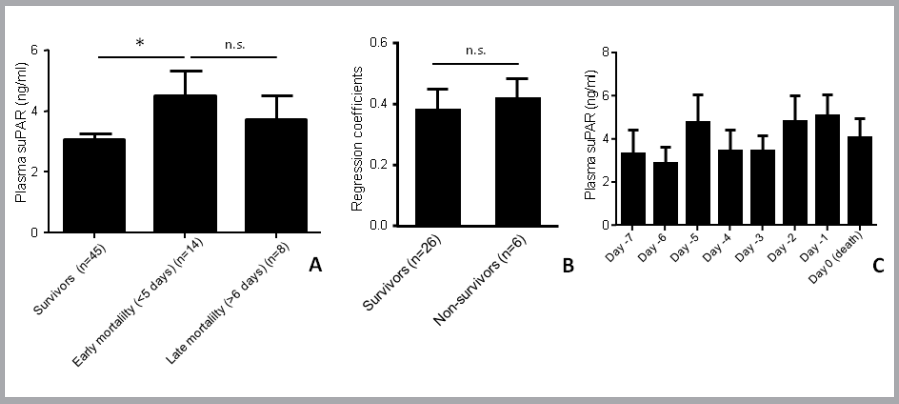


***Figure 2 – suPAR and survival*** *A. Initial plasma suPAR levels (determined at HEMS or ER) were significantly lower in survivors in comparison with non-survivors that died within 5 days after trauma. No differences in suPAR levels were observed between early and late mortality. Data are expressed as mean±SEM. * p<0.05 according to one way ANOVA with post-hoc Bonferroni's multiple comparison post-hoc test. B. Regression coefficients were similar in survivors and non-survivors according to unpaired student's T-test. C. Plasma suPAR concentrations in non-survivors. Day 0 indicating day of death, days -7 to -1 represent the days preceding death. No increase in suPAR preceding death was observed. There were no statistically significant differences between time points according to one way ANOVA.*

**Predictive value of early suPAR**

A receiver operating characteristic (ROC) curve was produced using the first obtained suPAR value to evaluate its predictive value for mortality (Figure 3). The area under the curve was 0.60 (95% CI 0.48 – 0.72). The optimal cut-off point (>3.66 pg/ml) resulted in a sensitivity of 50% (95% CI 28.2 – 71.8), specificity of 76.6% (95%CI 62.0 – 87.7), positive predictive value of 50% (95% CI 28.2 – 71.8) and negative predictive value of 76.6% (95%CI 62.0 – 87.7).

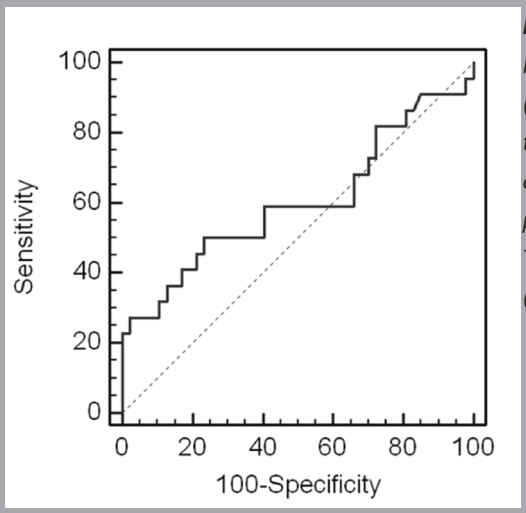


***Figure 3 – ROC curve suPAR*** *Receiver operating characteristic (ROC) curve analysis showing the utility of first plasma suPAR concentration as a prognostic test predicting death in trauma patients. The area under the curve was 0.60 (95% CI 0.48 – 0.72).*

**Relation between cytokines at ER and suPAR levels**

Median plasma cytokine levels at ER were: IFN-γ 3 pg/ml [IQR 3-3], IL-10 45 pg/ml [IQR 20–206], IL-6 25 pg/ml [IQR 8-68], IL-8 11 pg/ml [IQR 7-26], MCP-1 422 pg/ml [IQR 269-806], and TNF-α 6 pg/ml [IQR 3-12]. According to Pearson's correlation test after log transformation of cytokine values, plasma suPAR levels at time point ER correlated with plasma TNF-α measured at the same time point ($r=0.43$, $p<0.01$), while no correlation between other plasma cytokines and SuPAR levels at time point ER was found. However, pro-inflammatory cytokines IL-6, IL-8 and MCP-1 measured at the ER did correlate with suPAR levels at days 1, 3 and/or 5 (Table 2).

## Discussion

Herein, we show that plasma suPAR levels increase over time in trauma patients. Moreover, this study is the first to demonstrate that suPAR levels shortly after trauma are higher in non-survivors than in survivors, although predictive value for mortality was low and death itself was not preceded by an increase in suPAR. Furthermore, early increases in pro-inflammatory cytokine levels are related to increased suPAR levels at later time-points.

Several studies have demonstrated that suPAR is not as valuable as a single marker in clinical practice, mainly because of the lack of a clear cut-off value. Studies in (suspected) sepsis patients demonstrated ROC curves with an AUC between 0.5 and 0.75 and thus the value of suPAR in triage is unclear [6, 8]. This is in

| | ER suPAR | Day 1 suPAR | Day 3 suPAR | Day 5 suPAR |
|---|---|---|---|---|
| **ER TNF-α** | r=0.43<br>p<0.01<br>n=51 | r=0.08<br>p=0.60<br>n=38 | r=-0.02<br>p=0.89<br>n=23 | r=0.12<br>p=0.60<br>n=19 |
| **ER IL-6** | r=0.20<br>p=0.15<br>n=51 | r=0.17<br>p=0.28<br>n=38 | r=0.46<br>p=0.02<br>n=23 | r=0.47<br>p=0.04<br>n=19 |
| **ER IL-8** | r=0.18<br>p=0.20<br>n=51 | r=0.32<br>p=0.04<br>n=38 | r=0.48<br>p=0.01<br>n=23 | r=0.51<br>p=0.02<br>n=19 |
| **ER IL-10** | r=0.03<br>p=0.81<br>n=51 | r=0.11<br>p=0.49<br>n=38 | r=0.21<br>p=0.33<br>n=23 | r=0.26<br>p=0.28<br>n=19 |
| **ER MCP-1** | r=-0.03<br>p=0.82<br>n=51 | r=0.22<br>p=0.17<br>n=38 | r=0.41<br>p=0.05<br>n=23 | r=0.46<br>p=0.04<br>n=19 |
| **ER IFN-γ** | r=-0.01<br>p=0.95<br>n=47 | r=0.06<br>p=0.71<br>n=34 | r=-0.20<br>p=0.37<br>n=21 | r=0.10<br>p=0.69<br>n=16 |

***Table 2: Correlation between cytokine levels at the ER and suPAR levels at various time-points.*** *Grey boxes implicate significant correlations*

accordance with our results, as we demonstrate that the value of plasma suPAR concentration as a triage marker in individual patients is limited and its positive and negative predictive value is low. It is suggested that in sepsis or in patients with suspected infection at the ER, suPAR could be of value when combined with other biomarkers, which could also hold true for trauma patients [6, 8].

Of interest, we demonstrated a relation between early cytokine production and subsequent increase of suPAR levels. Patients with an initial high cytokine response, had high plasma suPAR concentrations later on during their hospital stay. This finding may indicate that the increase of suPAR over time could be mediated by the initial inflammatory response in trauma patients, although we cannot demonstrate causality from our dataset. In accordance, previous in vitro studies have demonstrated that neutrophils can release suPAR in response to stimulation with IL-8 or TNF-α [20] and endothelial cells after stimulation with IL-1β or PDGF [21]. Although the exact mechanism behind suPAR release has not yet been elucidated completely [22], based on these and our results, it seems possible

that cytokines play a role in this process. Nevertheless, the mediator responsible for the increased plasma suPAR concentration directly after trauma is unclear. Cytokines are unlikely to play a role in this initial increase, regarding the lack of correlation between cytokine levels and plasma suPAR at the ER. Moreover, in our patient cohort, relatively low plasma cytokine concentrations were found compared with other conditions, such as sepsis [23]. This might explain the relatively mildly increased suPAR levels (mean 4.1 ng/ml) in trauma patients at admission compared with those observed in patients with sepsis (median 8.9 [range 5.9-12.7] ng/ml) [24].

Our study has several limitations. Inherent to this kind of study, the number of patients that can be analyzed over time is limited as a substantial number of patients were lost to follow-up, for example because of death, transfer to another hospital or withdrawal from the study. However, the increase in SuPAR over time does not appear to result from the lost-to follow up, as the regression coefficients were positive in virtually all patients with a follow-up of at least three days. Moreover, the increasing trend in suPAR is also observed in the subgroup of patients that had a complete follow-up. Therefore, it appears unlikely that the increase in suPAR is the result of exclusion of less severely ill patients or patients that die during follow-up. Another weakness of the current study is the heterogeneity of the patients, which is inherent to the trauma patient population studied. Age could possibly have confounded our results. Various studies have assessed the relation between age and suPAR in several diseases, with contradictory results. Some demonstrate no correlation [25, 26], while others demonstrate higher [27,28] or lower [29] suPAR levels in elderly patients. We found a weak, but statistically significant correlation between age and suPAR. We do not think that that the increased suPAR levels in trauma patients are the result of the higher age of the patient cohort, also because suPAR levels increased over time in our cohort, while median age did not.

## Conclusions

In conclusion, early suPAR concentrations are higher in non-survivors than in survivors, and levels at later time-points are related to preceding cytokine levels. However, the predictive value of suPAR for mortality and therefore its clinical relevance is low. The steady increase over time related to the initial inflammatory response is not related to mortality.

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# Chapter 11

## General discussion and future perspectives

The immune system responds to invading pathogens, hallmarked by Pathogen Associated Molecular Patterns (PAMPs), but also to Danger Associated Molecular Patterns (DAMPs) that originate from the extracellular matrix or are released by injured, threatened, or dead cells [1-5]. This could be of great relevance in intensive care-related conditions, in which it is implicated that large amounts of DAMPs are released. Furthermore, critically ill patients often suffer from immune system-related complications [6], such as systemic inflammatory response syndrome (SIRS) or immunoparalysis. In the studies described in this thesis, the immunologic roles of DAMPs in critically ill patients were investigated. In this chapter, we discuss the findings of the studies performed, draw overall conclusions, and present future perspectives.

## Part I - DAMPs in critically ill patients

In the first part of this thesis, we demonstrated that DAMPs are released in patients suffering from trauma, cardiac arrest, leukemia, and sepsis. Moreover, we found that in trauma and cardiac arrest, DAMP release is associated with the development of immunoparalysis, while in leukemia no such relationship could be established. In patients with septic shock, DAMP release was associated with markers of inflammation, shock, and organ damage. Finally, we have shown that several pattern recognition receptors (PRRs), that can bind DAMPs, demonstrate synergistic or inhibitory interactions.

Although we have established a correlation between DAMPs and immunoparalysis in both trauma and post-cardiac arrest patients, the observational nature of our studies does not allow us to conclude that a causal relationship is present. Although DAMPs were released in trauma, cardiac arrest, leukemia, and sepsis, substantial differences in the extent of general DAMP release (reflected by nDNA levels) between those conditions were found (Figure 1). Sepsis and trauma patients display similar levels of nDNA, which are much higher compared with patients following cardiac arrest or those receiving chemotherapy treatment for leukemia. This difference appears feasible, taking the major impact on the entire body in both sepsis and trauma into account [7]. Although leukemic patients treated with chemotherapy display similar levels of circulating DAMPs as cardiac arrest patients, no relationship between DAMP release and immunoparalysis could be established in this specific group of patients. This difference might be explained by the profound changes in leukocyte phenotype and function due to the haematological malignancy. Differences in cell maturation, high percentages

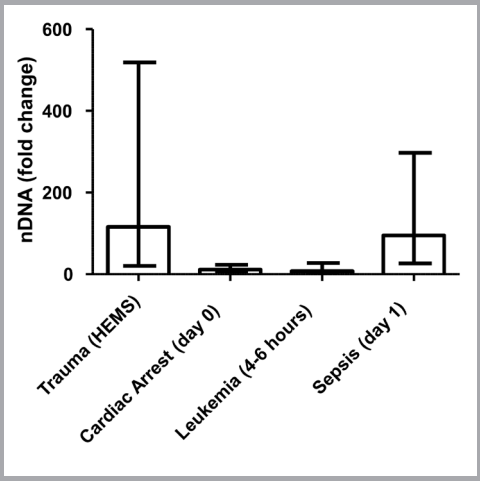


***Figure 1 – nuclear DNA in critically ill patients*** *Plasma levels of nuclear DNA (nDNA), a marker for general DAMP release, expressed as fold change to a calibrator sample in patients after trauma, cardiac arrest, chemotherapy in leukemia, and sepsis, at peak time points (pre-hospitally [on the trauma scene], within 24 hours after cardiac arrest, 4-6 hours after initiation of chemotherapy, and on the first day of norepinephrine therapy, respectively). Data are depicted as median±interquartile range.*

of blasts, and chemotherapy treatment directed at leukocyte destruction might influence the effect of DAMPs on immune cells. Moreover, although nDNA is assumed to be a marker for general DAMP release, as it is a major cell component that is released in case of cell damage, it is plausible that due to different mechanisms of damage (e.g. inflammation, ischemia or physical damage), variable combinations and ratios of different DAMPs are released. This underlines the fact that multiple complex processes take place simultaneously in critically ill patients, making it difficult to distinguish causality from epiphenomena and unravel underlying mechanisms in observational studies. Nevertheless, some studies suggest that there is a causal relationship between DAMP release and inflammation and that there might be a role for DAMP neutralization as a therapeutic intervention. For instance, in a recent murine study, administration of DNAses (to remove free nucleic acids from the circulation) resulted in decreased coagulation and inflammation, suppression of organ damage, and improved outcome in a cecal ligation and puncture sepsis model [8]. These findings imply that the presence of nucleic acids, such as nDNA and mtDNA described in our studies, could importantly impact prognosis and survival in sepsis patients. Moreover, the DAMP HSP70 has been shown to induce endotoxin tolerance in monocytes *in vitro* [9]. Along these lines, HSP70 was shown to downregulate TLR-4 expression and as such modulate the immune response to LPS and other TLR-4 ligands [10]. These findings suggest that this DAMP may play a direct role in suppression of the innate immune response *in vivo*, as observed in immunoparalysis. Future studies investigating inhibitors of specific DAMPs or their receptors could shed more light on the interaction between DAMPs and immunoparalysis. These data could

also indicate whether DAMP neutralization has therapeutic potential. It should however be kept in mind that neutralization or administration of a single DAMP could present an unclear picture of the *in vivo* situation, as in patients, a multitude of DAMPs are usually involved.

Of special interest in terms of possible therapeutic or preventive intervention is the early phase in trauma patients, as discussed in chapter 2. A promising study in restoring immunoparalysis by inhalation therapy of IFN-γ, suggests an intervention can safely improve outcome in patients with an impaired immune response following trauma [11]. Therapy was initiated days after hospital admission in this study. As we have demonstrated that immunoparalysis in trauma patients occurs already very early on, and median time to development of a hospital-acquired infection was only 6 days, the possibility to intervene in an earlier stage seems appealing. However, caution is warranted in this early phase, as it is hard to distinguish patients that could benefit from such a treatment and patients that could potentially harmed. For instance, immunostimulatory treatment could also result in an overwhelming pro-inflammatory response, leading to Systemic Inflammatory Response Syndrome (SIRS), which could ultimately result in shock, organ failure, and death [12]. This dilemma shows similarities with current paradigm in sepsis, in which it is believed that correctly timed immunostimulatory treatment could prevent deaths due to secondary infections, but only if correctly timed[6]. More studies focusing on the very early phase following trauma are crucial for further understanding of immunological pathways and mechanisms activated in this stage. For instance, more extensive knowledge is warranted on which DAMPs are released and to what extent, which DAMPs influence the immune response, and which immune cells and immune activation pathways are involved early after trauma. This data could help find suitable biomarkers that could identify patients that would benefit from immunostimulatory treatment.

As outlined above, DAMPs can have detrimental effects through inducing immunoparalysis. However, beneficial effects of some DAMPs have also been described. For example, neutralization of S100B exerted detrimental effects in an *in vitro* trauma model, indicating the protective properties of S100 proteins [13]. Moreover, rats subjected to CLP exhibited less sepsis-induced apoptosis in multiple organs when pre-treated with whole-body hyperthermia, which strongly induced HSP7014. Likewise, glutamine administration in rats resulted in increased expression of HSP70 and decreased mortality after CLP [15]. The mechanisms behind these protective effects remain largely unclear, but it could be speculated

that the same immunosuppressive effects which may result in development of immunoparalysis are involved can be beneficial in case of a too pronounced or sustained pro-inflammatory response. One could argue that from an evolutionary point of view, an immunological "shut-down" following major DAMP release could be of benefit to prevent an overwhelming pro-inflammatory response and subsequent organ damage. However, caution is warranted in translating these results obtained in animal models to a clinical setting. For instance, a wide variety of anti-inflammatory therapies showed very promising results in preclinical sepsis models, but none of these have eventually proved to be of benefit in clinical trials [20, 21].

As alluded to before and inherent to the mechanisms of release of DAMPs, we can assume that in many conditions on the ICU, multiple DAMPs are released simultaneously. In case of cell rupture or uncontrolled cell death, many cellular components are released, resulting in a broad range of different DAMPs in the plasma or surrounding tissue. Moreover, as the stimuli inducing active release of DAMPs show many similarities, such conditions will also result in the release of a multitude of DAMPs. As we have demonstrated in chapter 6 of this thesis, ligation of multiple pattern recognition receptors (PRRs) simultaneously can result in either synergistic or inhibitory interactions. Therefore, it appears likely that such interactions occur in vivo in case of DAMP release. Next to interaction on receptor level, other mechanisms of interaction between DAMPs could occur. Several studies have addressed this aspect of immune activation/inhibition by DAMPs. For example, nucleic acids as DAMPs are suggested to be dependent on the release of other DAMPs, e.g. HMGB1, and can also affect the activity of other DAMPs [22]. PRRs recognizing nucleic acids are generally present intracellularly, and internalization necessary for receptor-binding is stimulated by other DAMPs [22].

## Part II – DAMPs and ventilator-induced inflammation

The second part of this thesis comprises two studies on ventilator-induced inflammation, demonstrating that neither the mitochondrial DNA (mtDNA)/TLR9 pathway nor the inflammasome plays a role in this phenomenon.

The DAMP mtDNA has gained increasing attention in recent years [23-27]. Nevertheless, in the first part of this thesis, we did not find mtDNA to be released to a great extent or having considerable effects in the conditions studied. Subsequently, in chapter 7 we demonstrated in a murine ventilator-induced inflammation model, that mtDNA release and TLR9 signaling are not involved

mechanical ventilation-induced pulmonary inflammatory response. Combining those findings, it can be debated whether or not mtDNA/TLR9 signaling is a major contributor in critically ill patients. Several observational studies have shown that mtDNA levels in plasma of critically ill patients are increased [23, 24, 26, 28, 29], and animal studies on administration of exogenous mtDNA have demonstrated that mtDNA exerts pro-inflammatory effects [30, 31]. Although it appears likely that mitochondria and/or mtDNA are released as a result of a hit, several mechanisms preventing downstream effects could also play a role. A process known as mitophagy could result in swift clearance of mitochondria, even before cell death occurs [32]. Moreover, the bacterial-like appearance of mtDNA could result in rapid recognition and phagocytosis after release from damaged cells. Future studies on the role of mtDNA release in vivo are warranted. Even if this role appears minimal, measuring circulating mtDNA could still have value as a biomarker in clinical practice. For example, studies have indicated it has prognostic or diagnostic value in cancer [33, 34] and in traumatic brain injury [35].

As demonstrated in chapter 8, the inflammasome does not play a role in the mechanical ventilation-induced inflammatory response, and therefore interventions aimed at inflammasome inactivation might not have beneficial effects. Our study demonstrates that neutrophils are important in the pathophysiology of mechanical ventilation-induced inflammation, thereby representing a potential therapeutic target. Subjects of future research should include inhibition of serine proteases, which are neutrophil factors that can cleave pro-IL-1β, through administration of α1-antitrypsin (AAT) [36, 37]. Interestingly, patients with AAT deficiency demonstrate increased neutrophil influx [38], whereas AAT results in a reduction of neutrophil chemotaxis [39]. Furthermore, AAT has demonstrated promising results in animal studies on for example the treatment of gouty arthritis [40] and diabetes [41]. If AAT administration reduces neutrophil influx or serine protease activity following mechanical ventilation, it could possibly attenuate the ventilator-induced inflammatory response and reduce lung injury.

## Part III – Biomarkers in trauma

The third part of this thesis describes the results of two studies with biomarkers, intestinal fatty acid binding protein (iFABP) and soluble urokinase plasminogen activator receptor (suPAR), in trauma patients.
Using iFABP as a marker, we demonstrated that intestinal injury occurs immediately after trauma in patients with abdominal trauma, low blood pressure,

or low haemoglobin levels. Intestinal injury is suggested to be related to late (e.g. infectious) complications in trauma patients due to reduced intestinal barrier function and related increased bacterial translocation [42, 43]. In rare, but severe cases, acute mesenteric ischemia can occur, a serious condition with mortality rates up to 85%[44]. Thus, intestinal damage as found in trauma patients should be prevented where possible. Although resuscitation therapies in pre-hospital care and at the emergency room are directed towards retaining adequate blood pressure, extra attention should also be paid to low haemoglobin levels. Although transfusion is a common therapy for anemia, it also poses risks that should well be considered [45, 46]. Moreover, the optimal haemoglobin level for critically ill patients is still unclear, as such, the clinical benefits of targeting high haemoglobin levels remain to be determined.

We have also shown that, although the inflammatory marker suPAR is higher in patients that eventually did not survive trauma compared with survivors, the predictive value at the emergency room is low. This is in accordance with many other studies in several (intensive care) conditions, demonstrating similar results [47-51]. Overall, suPAR appears not a useful marker in diagnosis and/or prognosis at the ER, though several studies have suggested it could contribute in panels of biomarkers [47, 52]. However, this contribution is only minor, e.g. adding 0.04 to the AUC of the ROC curve compared with using only SAPS II for prediction of 30-day mortality in SIRS patients [52]. This suggests that the role for suPAR as a marker in clinical practice is limited.

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# Chapter 12

Summary
Nederlandse samenvatting

## Summary

The innate immune system is crucial in distinguishing between entities an immune response should be mounted to, and those it should ignore. Invading pathogens are recognized through detection of Pathogen Associated Molecular Patterns (PAMPs) by Pattern Recognition Receptors (PRRs). Following recognition, an immune response is initiated, accurately balanced between an overwhelming pro-inflammatory response that could result in too much "collateral damage" to the host and an anti-inflammatory response leaving the host susceptible for secondary infection.
Danger Associated Molecular Patterns (DAMPs) that are released by injured, threatened, or dead cells, or originate from the extracellular matrix, can also influence the immune system. This is of great relevance in critically ill patients, in whom trauma- or surgery-related cell damage, hypoxia, ischemia, and infections can result in extensive release of DAMPs. As many patients at the intensive care unit suffer from immune system-related complications, DAMPs could serve as markers for the prognosis of these patients and represent possible therapeutic targets. For example, neutralization of DAMPs could possibly revert or prevent detrimental effects, such as systemic inflammatory response syndrome (SIRS) or immunoparalysis. This thesis aims to shed light on the role DAMPs and the associated immune responses play in conditions frequently observed in critically ill patients. Apart from DAMPs, we studied the predictive properties of immunologically inactive markers in severe trauma patients admitted to the ICU.

In **chapter 1**, we provided an overview of several well-known DAMPs (High Mobility Group Box 1, heat shock proteins, s100 proteins, nucleic acids, and hyaluronan) and their effects on the immune system. Furthermore, we discussed the role of DAMPs as markers or therapeutic targets in several conditions frequently encountered in critically ill patients, such as sepsis, trauma, ventilator-induced lung injury, and cardiac arrest. Moreover, the aim and outline of this thesis was presented.

**Part I: DAMPs in critically ill patients**
The release of DAMPs can exert immunologic effects in critically ill patients and have an impact on outcome. Moreover, simultaneous ligation of multiple PRRs by various DAMPs, which is likely to occur in critically ill patients, could have different effects than ligation of single receptors.
A suppressed immune system, known as "immunoparalysis" in the sepsis research

field, may contribute to the increased vulnerability towards hospital-acquired infections in polytrauma patients. In **chapter 2** we studied the time course of immunoparalysis in trauma patients, starting from the pre-hospital phase until 10 days after the trauma and the relationship between markers of immunoparalysis and the development of infections in these patients. Moreover, the involvement of DAMPs was examined. We conclude that immunoparalysis, characterized by decreased HLA-DR expression and dysregulated cytokine production, is apparent within minutes/hours following trauma and appears to be related to release of DAMPs nDNA and HSP70 and production of large amounts of anti-inflammatory IL-10 in the pre-hospital phase. Moreover, further decreasing HLA-DR expression in the first days after trauma is associated with an increased susceptibility to develop infections.

An increased risk of infection has also been reported in cardiac arrest patients that are admitted to the ICU following out-of-hospital resuscitation. Therefore, in **chapter 3** we investigated whether immunoparalysis develops following cardiac arrest and whether the release of DAMPs could be involved. In accordance with the results of our study in trauma patients described in chapter 2, we showed that release of DAMPS nDNA, HSP70, and EN-RAGE during the first days after cardiac arrest is associated with the development of immunoparalysis, characterized by a more anti-inflammatory cytokine response to LPS ex vivo. This could contribute to the increased susceptibility towards infections in cardiac arrest patients.

Chemotherapy used to treat haematological malignancies may result in the release of large amounts of DAMPs. Furthermore, it is well known that patients treated with chemotherapy are extremely vulnerable for infections, primarily due to chemotherapy-induced immune cell destruction. However, the function of remaining immune cells might also be compromised because of immunoparalysis and DAMP release might be involved. In **chapter 4**, we studied DAMP release and markers of immunoparalysis before and up to 8 days after initiation of chemotherapy. We found that in the early phase following chemotherapy, DAMP levels are profoundly increased, however, this does not induce immunoparalysis in this specific group of patients

In sepsis patients, a relationship between release of nucleic acids and mortality has been demonstrated. However, the intermediate factors and/or mechanisms contributing to this relation are largely unknown. In **chapter 5**, our aim was to determine whether plasma levels of nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) are related to the markers of inflammation, shock, and organ damage in septic shock patients. We showed that during the first 5 days after onset of septic shock, nDNA, but not mtDNA levels consistently correlated with plasma cytokine

concentrations as well as with shock-related parameters (noradrenalin infusion rate and heart rate) and markers of organ damage (total bilirubin and creatinin). These results may explain the underlying mechanisms behind the relationship between plasma levels of nucleic acids and mortality in sepsis patients.
In critical illness, multiple DAMPs are released simultaneously on many occasions, thereby triggering multiple PRRs at the same time. Interactions between those receptors could therefore be important. In **chapter 6**, we performed a systematical analysis of the interactions between PRR signaling pathways. A number of PRR-dependent signaling interactions were found to be consistent, both between individuals and with regard to multiple cytokines. The combinations of TLR2 and NOD2, TLR5 and NOD2, TLR5 and TLR3, and TLR5 and TLR9 acted as synergistic combinations. Surprisingly, inhibitory interactions between TLR4 and TLR2, TLR4 and Dectin-1, TLR2 and TLR9 as well as TLR3 and TLR2, were observed. These consistent signaling interactions between PRR combinations may represent promising targets for immunomodulation and vaccine adjuvant development, for example to activate the immune response by using multiple PRRs. Moreover, they can have implications for conditions in which multiple DAMPs and/or PAMPs are thought to play a role.

**Part II – DAMPs and ventilator-induced inflammation**

Mechanical ventilation is an essential part of perioperative and intensive care medicine. However, mechanical ventilation can induce an inflammatory response in the lungs which compromises pulmonary function, and may cause injury to other organs. DAMPs are implicated to play a role in the ventilator-induced inflammatory response. In **chapter 7**, using a mouse model of ventilator-induced inflammation, we demonstrated that mechanical ventilation with either low or high tidal volumes elicits a pulmonary inflammatory response, but does not result in mtDNA release into the pulmonary compartment. Furthermore, using knockout mice, we showed that the receptor for mtDNA, TLR9, does not play a role in the ventilator-induced inflammatory response. In conclusion, the mtDNA/TLR9 pathwya does not play a role in ventilator-induced inflammation.
Previous studies have shown that the pro-inflammatory cytokine IL-1β plays a pivotal role in the MV-induced inflammatory response. Cleavage of the inactive precursor pro-IL-1β to form bioactive IL-1β is mediated by several types of proteases, of which caspase-1, activated within the inflammasome, is the most important. In **chapter 8**, we demonstrated in a series of mouse experiments that the inflammasome/caspase-1 appears not to be involved in IL-1β processing in the ventilator-induced inflammatory response, which is driven by DAMPs.

The attenuated inflammatory response observed in ventilated anti-KC treated and neutropenic mice suggests that IL-1β processing in mechanical ventilator-induced inflammation is mainly mediated by neutrophil factors.

**Part III – Biomarkers in trauma**

Although DAMPs could serve as biomarkers in the ICU, such as in severe trauma patients, immunologically inactive biomarkers could help determine prognosis and treatment options as well.

One of these markers, intestinal Fatty Acid Binding Protein (iFABP), is a marker of intestinal injury. In **chapter 9**, we showed that plasma iFABP levels are increased immediately after trauma in patients with abdominal trauma, low MAP, or low Hb, and are related to the severity of the trauma. As intestinal injury is suggested to be related to late complications, such as multiple organ dysfunction syndrome (MODS) or sepsis in trauma patients, more attention to prevent intestinal damage following trauma could be of benefit in these patients.

Soluble urokinase-type plasminogen activator (suPAR) has been identified as a marker for immune activation and has predictive value in multiple groups of patients. In **chapter 10**, we describe that in trauma patients, the first obtained plasma suPAR concentrations (measured before hospital admission or at the emergency department) are associated with mortality, although its predictive value is low. Furthermore, suPAR concentrations increase over time in these patients, and levels were related to the innate immune response observed in the ER. These results indicate that suPAR is an innate immune response-induced biomarker in trauma patients, but its clinical applicability is deemed low.

In conclusion, our major findings are that DAMPs are released in trauma, cardiac arrest, leukemia and sepsis. DAMP release is associated with the development of immunoparalysis in trauma and cardiac arrest, but not in leukemia, and with markers of inflammation, shock, and organ damage in septic shock. Mitochondrial DNA as a DAMP acting via TLR9 does not appear to play a role in the conditions studied, despite increasing attention in the past years. Future studies should focus on elucidating the exact (causal?) relationship between DAMPs and immunoparalysis, and the possible treatment options related. The pre-hospital phase in trauma, and possible biomarkers to identify patients that could benefit from treatment, should get extra consideration in this respect.

## Nederlandse samenvatting

Ernstig zieke patiënten op de intensive care hebben een vergrote kans op het ontwikkelen van infecties, bovenop hun bestaande aandoening. Hierdoor bestaat een grotere kans op complicaties, langer verblijf in het ziekenhuis of zelfs overlijden. Het afweersysteem is verantwoordelijk voor de bescherming tegen organismen die een infectie kunnen veroorzaken. Wanneer het niet goed functioneert, ontstaat een groter risico op infecties. Daarom is het van belang om zo goed mogelijk te begrijpen hoe het afweersysteem werkt bij deze ernstig zieke patiënten.
Het aangeboren afweersysteem is cruciaal voor het onderscheiden van zaken waartegen een afweerreactie moet worden geïnitieerd van zaken die genegeerd dienen te worden. Patroonherkenningsreceptoren herkennen binnendringende ziekteverwekkers door de detectie van Pathogen Associated Molecular Patterns (PAMPs), zoals de celwand van bacteriën, DNA van virussen en sporen van schimmels. Na herkenning start een afweerreactie, waarbij nauwkeurig het evenwicht bewaard wordt tussen een overweldigende ontstekingsreactie die kan leiden tot teveel schade aan de patiënt en een anti-ontstekingsreactie die de patiënt vatbaar kan maken voor infecties. Naast deze PAMPs beïnvloeden gevaarsignalen (Danger Associated Molecular Patterns, DAMPs) - vrijkomend uit gewonde, bedreigde of dode cellen of weefsels van de patiënt zelf - eveneens het afweersysteem. Dit kan gevolgen hebben voor ernstig zieke patiënten, bij wie trauma- of chirurgiegerelateerde celschade, zuurstoftekort, bloedtekort of infecties kunnen leiden tot het vrijkomen van veel DAMPs. Aangezien veel patiënten op de intensive care aan het afweersysteem gerelateerde complicaties krijgen, kunnen DAMPs als markers dienen voor de prognose van deze patiënten. Bovendien zouden ze als mogelijke therapeutische aangrijpingspunten kunnen dienen, bijvoorbeeld wanneer neutralisatie van DAMPs de nadelige effecten, zoals ontsteking door het hele lichaam of slecht functioneren van het afweersysteem, op kunnen heffen of voorkomen. Dit proefschrift heeft als doel om licht te werpen op de rol die DAMPs en de bijbehorende afweerreacties spelen bij aandoeningen die vaak voorkomen op de intensive care. Daarnaast hebben we de voorspellende waarde van markers die niet als DAMP werken bij patiënten met een ernstig trauma bestudeerd.

In **hoofdstuk 1** hebben we een aantal bekende DAMPs (High Mobility Group Box 1, heat shock eiwitten (HSPs), s100 eiwitten, nucleïnezuren, en hyaluronzuur) en hun effecten op het afweersysteem beschreven. Verder hebben we de rol

van DAMPs als markers of therapeutische aangrijpingspunten bij patiënten met sepsis, trauma, beademingsgeïnduceerde longschade en hartstilstand besproken. Bovendien hebben we het doel en de opzet van dit proefschrift gepresenteerd.

**Deel I: DAMPs bij ernstig zieke patiënten**

Het vrijkomen van DAMPs kan effecten hebben op het afweersysteem van ernstig zieke patiënten en daardoor de uitkomst van hun ziekte beïnvloeden. Bovendien kan gelijktijdige binding van meerdere receptoren door verschillende DAMPs, iets wat zeer waarschijnlijk gebeurt bij ernstig zieke patiënten, een ander effect hebben dan de binding van slechts een enkele receptor.

Een onderdrukt afweersysteem, ook wel "immuunparalyse" genoemd, kan bijdragen aan de verhoogde gevoeligheid van patiënten na een ernstig ongeval (traumapatiënten) voor ziekenhuisinfecties. In **hoofdstuk 2** bestudeerden we het tijdsverloop van immuunparalyse bij traumapatiënten vanaf de pre-hospitale fase (direct na trauma, "op straat") tot 10 dagen na het trauma. Ook keken we naar de relatie tussen de markers van immuunparalyse en het ontstaan van infecties bij deze patiënten. Bovendien werd de betrokkenheid van DAMPs hierbij onderzocht. We concludeerden dat immuunparalyse, gekenmerkt door verminderde expressie van HLA-DR (een belangrijk eiwit voor het afweersysteem) en ontregelde productie van ontstekingseiwitten, binnen minuten/uren na trauma ontstaat. Bovendien bleek de immuunparalyse gerelateerd aan het vrijkomen van DAMPs en de productie van grote hoeveelheden van het anti-ontstekingseiwit IL-10 al voor de patiënt in het ziekenhuis is. Tenslotte bleek het nog verder afnemen van de HLA-DR expressie in de eerste dagen na het trauma geassocieerd met een toegenomen vatbaarheid voor infecties.

Ook bij patiënten die na een hartstilstand en daaropvolgende reanimatie buiten het ziekenhuis zijn opgenomen op de intensive care, wordt een verhoogd risico op infecties gemeld. Daarom hebben we in **hoofdstuk 3** onderzocht of immuunparalyse ontwikkelt na een hartstilstand en of het vrijkomen van DAMPs hierbij betrokken is. Overeenkomstig met de resultaten van onze studie bij traumapatiënten, toonden we aan dat afgifte van DAMPs tijdens de eerste dagen na hartstilstand is geassocieerd met de ontwikkeling van immuunparalyse. De immuunparalyse werd gekenmerkt door een anti-ontstekingsreactie na blootstelling van het bloed aan een deel van een bacterie (LPS) in het laboratorium. Dit kan bijdragen tot de verhoogde gevoeligheid voor infecties bij patiënten na een hartstilstand.

Chemotherapie die gebruikt wordt voor het behandelen van bijvoorbeeld leukemie kan resulteren in het vrijkomen van grote hoeveelheden DAMPs.

Bovendien is het bekend dat patiënten behandeld met chemotherapie uiterst gevoelig voor infecties zijn, met name door de vernietiging van afweercellen door de chemotherapie. Echter, de functie van de resterende afweercellen zou ook aangedaan kunnen zijn als gevolg van immuunparalyse, waarbij het vrijkomen van DAMPs mogelijk een rol zou kunnen spelen. In **hoofdstuk 4** bestudeerden we het vrijkomen van DAMPs en de markers voor immuunparalyse vóór en tot 8 dagen na de start van de chemotherapie. We vonden dat in de vroege fase na chemotherapie behoorlijke hoeveelheden DAMPs aanwezig zijn. Dit resulteerde echter niet in immuunparalyse bij deze specifieke groep patiënten.
Bij patiënten met bloedvergiftiging (sepsis) is eerder een relatie tussen het vrijkomen van nucleïnezuren, zoals DNA, en sterfte aangetoond. De factoren en/of mechanismen die bijdragen aan deze relatie zijn echter grotendeels onbekend. In **hoofdstuk 5** onderzochten we of de plasmaspiegels van nucleair DNA (nDNA) en mitochondrieel DNA (mtDNA) gerelateerd zijn aan de markers van ontsteking, shock en orgaanschade bij patiënten met septische shock, een ernstige vorm van bloedvergiftiging. We toonden aan dat gedurende de eerste 5 dagen na het begin van septische shock de hoeveelheid nDNA, maar niet de hoeveelheid mtDNA, consistent gecorreleerd was met de hoeveelheid ontstekingseiwitten in het bloed, alsook met shock-gerelateerde parameters en markers van orgaanschade. Deze resultaten zouden de onderliggende mechanismen kunnen verklaren van de eerder aangetoonde relatie tussen plasmaniveaus van nucleïnezuren en sterfte in sepsispatiënten.
Bij ernstig zieke patiënten komen meerdere DAMPs gelijktijdig vrij, waarbij meerdere receptoren tegelijk geactiveerd worden. Mogelijke interacties tussen deze receptoren zouden daarom belangrijk kunnen zijn. In **hoofdstuk 6** hebben we een systematische analyse van de interacties tussen receptorsignaalroutes uitgevoerd. Een aantal interactieroutes tussen receptoren bleken consistent te zijn, zowel tussen individuen als tussen meerdere ontstekingseiwitten. De combinaties van receptoren TLR2 en NOD2, TLR5 en NOD2, TLR5 en TLR3 en TLR5 en TLR9 werden geïdentificeerd als synergistische combinaties. Bij het stimuleren van deze combinaties werden dus meer ontstekingseiwitten geproduceerd dan verwacht zou worden wanneer de individuele reacties bij elkaar opgeteld zouden worden. Opvallend genoeg werd een remmende wisselwerking waargenomen tussen TLR4 en TLR2, TLR4 en Dectin-1, TLR2 en TLR9 en TLR3 en TLR2. Deze combinaties resulteerden dus juist in minder ontstekingseiwitten die op basis van de som van beide individuele reacties verwacht zou worden. Deze consistente signaleringsinteracties tussen receptorcombinaties kunnen veelbelovende doelen voor therapeutisch ingrijpen op de afweerrespons en de ontwikkeling

van vaccinadjuvants vormen. Een vaccinadjuvant kan gebruikt worden om de afweerrespons sterker te maken, in dit geval via het stimuleren van meerdere receptoren. Bovendien kunnen deze resultaten gevolgen hebben voor onderzoek naar ziektebeelden waarin meerdere DAMPs en/of PAMPs geacht worden een rol spelen.

**Deel II - DAMPs en beademingsgeïnduceerde ontsteking**

Kunstmatige beademing is een essentieel onderdeel van zorg op de operatiekamer en de intensive care. Kunstmatige beademing kan echter een ontstekingsreactie in de longen induceren, die longfunctie kan verminderen en uiteindelijk ook schade in andere organen kan veroorzaken. Het is bekend dat DAMPs een rol spelen bij het ontstaan van de beademingsgeïnduceerde ontstekingsreactie. In **hoofdstuk 7** bestudeerden we specifiek de rol van mtDNA en de receptor TLR9 bij het ontstaan van beademingsgeïnduceerde ontsteking. Met behulp van een experimenten in muizen toonden we aan dat kunstmatige beademing met kleine of grote ademteugen een ontstekingsreactie in de long veroorzaakt, maar niet resulteert in het vrijkomen van mtDNA in de longen. Bovendien toonden we met behulp van genetisch gemodificeerde muizen zonder deze receptor aan dat de receptor voor mtDNA, TLR9, geen rol speelt bij de beademingsgeïnduceerde ontsteking. We concluderen dus dat de mtDNA/TLR9 route geen rol speelt bij beademingsgeïnduceerde ontsteking.

In eerder onderzoek is aangetoond dat het ontstekingseiwit IL-1β een centrale rol speelt bij beademingsgeïnduceerde ontsteking. Het inactieve pro-IL-1β kan door verschillende eiwitten omgezet worden in het bioactieve IL-1β. De belangrijkste daarvan is caspase-1, dat wordt geactiveerd in het inflammasoom. In **hoofdstuk 8** hebben we in een reeks muisexperimenten aangetoond dat bij beademingsgeïnduceerde ontsteking, die wordt aangedreven door DAMPs, het inflammasoom/caspase-1 niet betrokken is bij de activatie van IL-1β. Vervolgexperimenten toonden aan dat IL-1β activatie bij beademingsgeïnduceerde ontsteking voornamelijk wordt gemedieerd door neutrofielen, specifieke afweercellen.

**Deel III - Biomarkers in trauma**

Biomarkers zijn factoren die iets kunnen zeggen over de prognose van of geschikte therapie voor patiënten. Ook stoffen die niet als DAMP werken kunnen als biomarker dienen.

Eén van deze markers, het vetzuurbindend eiwit van de darm (intestinal Fatty Acid Binding Protein, iFABP), is een marker van darmschade. In **hoofdstuk 9** hebben we

aangetoond dat het iFABP niveau in plasma onmiddellijk na trauma is verhoogd bij patiënten met trauma aan de buik, een lage bloeddruk of laag hemoglobine, en verband houdt met de ernst van het trauma. Aangezien darmschade gerelateerd lijkt te zijn aan late complicaties (bijv. orgaanfalen of sepsis) bij traumapatiënten, zou meer aandacht voor de preventie van darmschade na trauma van nut kunnen zijn bij deze patiënten.

Oplosbare urokinase-type plasminogeen activator receptor (Soluble urokinase-type plasminogen activator receptor, suPAR) is geïdentificeerd als een marker die voorspellende waarde heeft in verschillende patiëntenpopulaties. In **hoofdstuk 10** beschrijven we dat bij traumapatiënten de suPAR concentratie die kort na trauma werd gemeten, geassocieerd is met sterfte. De voorspellende waarde is echter laag. Verder zien we dat de suPAR concentratie met de tijd stijgt bij deze patiënten en dat de niveaus gerelateerd waren aan de hoeveelheid ontstekingseiwitten gemeten op de spoedeisende hulp. Deze resultaten geven aan dat suPAR een door het aangeboren afweersysteem geïnduceerde biomarker is bij traumapatiënten, waarvan de klinische toepasbaarheid echter laag wordt geacht.

Samengevat zijn onze belangrijkste bevindingen dat DAMPs vrijkomen bij trauma, hartstilstand, leukemie en sepsis. DAMP afgifte wordt geassocieerd met de ontwikkeling van immuunparalyse bij trauma en hartstilstand (maar niet bij leukemie) en markers van ontsteking, shock en orgaanschade bij septische shock. Mitochondrieel DNA als DAMP die werkt via TLR9 blijkt geen rol te spelen in de bestudeerde condities, ondanks de toenemende aandacht voor deze DAMP in de afgelopen jaren. Toekomstig onderzoek moet zich richten op het ophelderen van het precieze (oorzakelijke?) verband tussen DAMPs en immuunparalyse, en de mogelijke therapeutische aangrijpingspunten die daaraan gerelateerd zijn. De pre-hospitale fase na trauma, en mogelijke biomarkers om patiënten die zouden kunnen profiteren van de behandeling te identificeren, moeten extra aandacht krijgen in dit opzicht.

# Chapter 13

Dankwoord
Curriculum Vitae
List of publications

Beste lezer,

Na het lezen van dit proefschrift, zult u zich vanzelfsprekend realiseren dat ik dit alles onmogelijk in mijn eentje gedaan kan hebben. Onderzoek is ondanks sommige eenzame momenten toch een teamsport. Ons "team" heeft de afgelopen jaren leerzaam, gezellig en bijzonder gemaakt. Ik ben trots op het resultaat, maar bovenal op hoeveel ik heb mogen leren en meemaken. Ik wil dan ook iedereen die op wat voor manier dan ook een bijdrage heeft geleverd aan dit proefschrift ontzettend bedanken. Een aantal mensen zou ik hierbij specifiek willen noemen.

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Als student heb ik tijdens mijn hoofdvakstage enorm veel geleerd op het laboratorium experimentele interne geneeskunde van professor Netea en professor Joosten. Mihai en Leo, bedankt voor het nog verder aanwakkeren van mijn passie voor immunologie. Gelukkig dachten jullie aan Gert Jan Scheffer, toen ik bij jullie aanklopte voor mogelijkheden om te promoveren. Bedankt

ook voor alle experimenten die ik op jullie lab heb mogen uitvoeren en voor de overleggen die met name het VILI onderzoek verder hebben geholpen. Ook op de cytokinemeetings waar ik altijd welkom was, heb ik ontzettend veel input gekregen. Tijdens mijn presentaties op deze besprekingen was ik ook blij dat professor van der Meer de volledige medisch-wetenschappelijke literatuur uit het hoofd leek te kennen en altijd weer waardevolle suggesties voor vervolgstudies deed. Jos, ik ben vereerd dat je ondanks het emeritaat op wilde treden als voorzitter van mijn manuscriptcommissie.

Professor van der Hoeven en Annemiek Broods hebben mij, als anesthesie-onderzoeker, de mogelijkheid gegeven onderzoek te doen op de Intensive Care. Ik heb mij zeer welkom gevoeld op mijn werkplek op de IC en weet zeker dat mijn proefschrift niet hetzelfde zou zijn geweest zonder deze samenwerking. Hans, bedankt voor jouw visie op onderzoek en de klinische relevantie die je nooit uit het oog verliest.

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De Intensive Care artsen, verpleegkundigen en ander personeel zijn eveneens van groot belang geweest voor mijn proefschrift. Fijn dat ik steeds weer langs mocht komen met vragen, of om alweer een buisje bloed af te nemen.
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De medewerkers van de afdeling Anesthesiologie hebben op allerlei manieren mijn onderzoek gefaciliteerd. Het onderzoek op de afdeling wordt steeds beter, ik denk dat we daar allemaal trots op mogen zijn.

Ook de co-auteurs van de stukken in dit proefschrift, Astrid Hoedemaekers, Maarten van den Berg, Arjan van Laarhoven, Nicole Blijlevens, Özcan Sir, Michael Edwards, Jeroen van der Laak, Theo Plantinga, Gosse Adema, Selina van der Wal, Stephanie Janssen, Pauline Maassen, Carmen de Jong en Aaron John wil ik graag bedanken voor hun bijdrage aan dit proefschrift en de prettige samenwerking.

Gedurende mijn promotie heb ik veel studenten begeleid. Van allemaal heb ik nieuwe dingen geleerd over begeleiden, communiceren of lesgeven. Ik ben hen natuurlijk dankbaar voor hun hulp bij de inclusie van patiënten en laboratoriumwerk, maar nog veel meer voor deze belangrijke lessen.

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Beste ……..……., jouw bijdrage aan dit proefschrift is niet in woorden uit te drukken. Dankjewel!

## Curriculum Vitae

Kim Timmermans werd geboren op 11 april 1987 te Loon op Zand. Na het behalen van haar VWO diploma aan het Sint Odulphuslyceum te Tilburg begon zij in 2004 aan de studie Biomedische Wetenschappen aan de Radboud Universiteit Nijmegen. Zij koos hierbij voor het hoofdvak Humane Pathobiologie, het bijvak geneesmiddelenonderzoek en een aantal keuzevakken immunologie en infectieziekten. In het kader van de studie werd een viertal stages gelopen. De bachelorstage, met als onderwerp de calcium transporter TRPV5, werd uitgevoerd onder supervisie van Prof. Bindels, Prof. Hoenderop en Dr. Renkema op de afdeling celfysiologie van het Radboud Institute for Molecular Life Sciences (RIMLS). In het kader van het bijvak geneesmiddelenonderzoek liep Kim stage op de afdeling farmacologie en toxicologie van het RIMLS en werd hierbij gesuperviseerd door Prof. Russell en Dr. Wittgen. Tijdens dit project bestudeerde zij het transporteren van antibiotica uit cellen door multi-drug resistance proteins. Vervolgens heeft zij aan de Universitas Muhammadiyah te Yogyakarta, Indonesië, onder begeleiding van Dr. Zulhah, een onderzoek uitgevoerd naar mogelijke behandelingen van anemie. Kim sloot haar studie af met een stage op de afdeling experimentele interne geneeskunde van het Radboudumc, waar zij onder begeleiding van Prof. Netea en Dr. Plantinga de interacties tussen verschillende pattern recognition receptors bestudeerde.

Na het behalen van het masterdiploma begon Kim in december 2009 als junior onderzoeker aan de afdeling pathologie van het UMC Utrecht. Hier deed zij onderzoek naar de mechanismen van cardiac allograft vasculopathy na harttransplantatie. In februari 2011 startte zij vervolgens met een promotie-onderzoek op de afdelingen Anesthesiologie en Intensive Care van het Radboudumc. De onderzoeksresultaten staan in dit proefschrift beschreven en werden bovendien gepresenteerd op verscheidene nationale en internationale congressen, wat onder meer resulteerde in prijzen voor beste presentatie op de wetenschapsdagen van de Nederlandse vereniging voor Anesthesiologie in 2013 en 2015.

Kim woont samen met Emile Brink en hun twee zoons Jasper en Wessel in Lent.

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- Timmermans K, Kox M, Vaneker M, Pickkers P, Scheffer GJ, Release of mitochondrial DNA and TLR9 signaling is not involved in mechanical ventilation-induced inflammation, submitted

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